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Evaluation of DNA Damage in Fish and Aquatic Insects Induced By Environmental Pollutants in River Nile

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Abstract: The development of comet assay for aquatic organisms is of particular relevance in light of the importance of fisheries testing for environmental pollutants. Various tests in organisms have been utilized for the detection and identification of toxic substances in the air, water and soil. In the present study, the comet assay was applied on fish and aquatic insects to conduct an environmental assessment of River Nile. The collected samples from the mixed point of waste water represented the highest degree of DNA damage concerning damage stages and comet% followed by the mixed point of agriculture drain when compared with samples collected from control site. Results of DNA damage by one way ANOVA analysis of tail moment of fish and aquatic insects collected from this study demonstrates the potential application of the comet assay to different aquatic sites were not significantly different from samples collected from control site (P= 0.08). While when analyzing other comet parameters (comet% and tail length) samples were significantly different (P= 0.04). The results suggested a genotoxicity of the aquatic environment at River Nile and that the comet assay in fish and aquatic insects provided adequate sensitivity to be utilized as a tool in the monitoring of water pollution and environmental risk assessment.

Key words: Aquatic insects · Biomarkers · Comet assay · Genotoxicity · DNA damage

INTRODUCTION

River Nile is the predominant source of fresh water in Egypt. Water Pollution was considered to be one of the most dangerous hazards affecting Egypt. Pollution in the River Nile System (main stream Nile, drains and canals) increased in the past few decades because of population increase; several new irrigated agriculture projects and other activities along the Nile [1]. Ali *et al*. [2] studied the water quality of the River Nile, Egypt and concluded that the River body received big quantities of domestic, industrial and agricultural wastes. As a result there was a continuous deterioration of water quality, because of not achieving a natural cleaning action. They used the biological and chemical characters of the River Nile to evaluate the trophic and autotrophic state of the River. The relationship between bacterial indicators with each other and with chemical variable and phytoplankton biomass revealed that the River Nile has become a pool for pumping domestic, industrial and agricultural effluents.

The utilization of fishes as bio-indicators of pollutant effects is being more and more used, since such practice can help to detect possible environmental problems. Results obtained in assays carried out with fishes can be useful for the evaluation of environmental presence of substances potentially teratogenic and carcinogenic in human beings [3]. Among the tests for genotoxicity, the micronucleus test has been widely utilized in fish to determine exposure to water pollutants, in the environment as well as under experimental laboratory conditions [4]. The single cell gel electrophoresis (SCGE) assay, commonly called the comet assay, is a genotoxicity test able to detect DNA damage induced by alkylating,

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intercalating and oxidizing agents [5, 6]. The comet assay has been used as an important tool for monitoring genotoxicity in aquatic environments. For this purpose, fish are used as a test organism in which it is possible to detect DNA damage induced by direct mutagens and pro-mutagens in both fresh and salt water [7]. This technique has also been employed in the determination of the genotoxic potential of water resources such as rivers and lakes. Meanwhile, the comet assay has been proposed as a tool to monitor genotoxicity in ocean and continental waters, utilizing fish for the detection of DNA damage induced by direct-acting mutagens and pro-mutagens dissolved in the water as well as environmental analysis of water samples [8]. In the present study, genotoxic effects were evaluated in aquatic insects and fish by using the alkaline comet assay for transmission of some zoonotic bacteriological pollutants in the River Nile at El Qanater and El Mansouria Canal (branch from the River Nile) Egypt.

MATERIALS AND METHODS

Sampling Sites and Collection: Twelve runs were collected monthly from 3 different types of water from River Nile as follows: The main stream of River Nile was considered as control (reference point) from El Qanater, Qalubya Governorate; River Nile mixed with agriculture drainage and River Nile mixed with sewage drainage from El-Mansouria canal (Giza Governorate).

Fish and Aquatic Insects: Aquatic insects were collected by sweeping the water with D-framed net which was the most common method. Then samples were transferred in ice box to the laboratory for identification and genetic examination. Fish were also collected from the same places as aquatic insects and transferred alive to the laboratory according to Saleh *et al*. [9].

The collected samples included aquatic insects and fish were identified and the most common insects species were: *Appasus urinator* (Family: Belostomatidae, oedrer: Hemiptera) and *Hydaticus leander* (family: Dytiscidae, order: Coleoptera) collected from the mixed point of agriculture drain. *Eristalis* sp. (family: Syrphidae, order: Diptera) and *Stratiomys sp.*(family: Stratiomyidae, order: Diptera) collected from the mixed point of wastewater. And *Appasus urinator* Family: Belostomatidae, oedrer: Hemiptera) and *Enallagma vansomereni* (family: Coenagriidae, order: Odonata) collected from the main stream of River Nile before branches. The collected fish samples from the mixed point of agriculture drain and the main stream of River Nile before branches were identified as: *Oreochronicus nilonica* (Nile Tilapia) (family: Cichlidae, order: Perciformes) and from the mixed point of wastewater: *Silurus triostegus* (Cat fish) (family: Siluridae, order: Siluriformes).

Single Cell Gel Electrophoresis (Comet Assay): The comet assay was performed under alkaline conditions essentially following the procedure of Singh *et al*. [10] with a slight modification. Tissue samples were taken from aquatic insects and fish muscle, homogenized in 1 mL cold HBSS (20 mM EDTA / 10% DMSO). 10 µl of cell suspension (≈2×10^5 cells) was embedded in 90 µl of 0.65% low-melting agarose layers on a Star-frost microscope slide, pre-coated with 0.65% normal melting agarose. After 20 min of solidification at 4°C, a third layer of 120 µl of 0.65% low-melting agarose was placed on top and left at 4°C for an additional period of 20 min to allow solidification. The cells were then lysed by immersing the slides overnight in a freshly prepared lysis solution (2.5M NaCl, 100mM EDTA, 10mM Tris, 1% Triton X-100, 10% DMSO, pH 10.0) at 4°C. After lysis, the slides were washed in cold water (3×) for 15 min and placed on a horizontal gel electrophoresis tray containing freshly prepared electrophoresis buffer (1mM EDTA, 300mM NaOH, pH 13.0) for 20 min to allow DNA unwinding. Electrophoresis was then carried out at 20V and at a starting current of 300mA for 20 min. Thereafter, the slides were neutralized with 0.4M Tris pH 7.5 for 15 min (3×), fixed in ethanol and dried. The slides were stained with 60 µl of 20µg/ml Ethidium bromide solution and viewed under a fluorescent microscope using a U-MNG filter (Olympus).

Image Analysis: The alkaline comet assay allows for detection of DNA damage occurring as single strand breaks (SSBs) by measuring the migration of DNA fragments from the nucleoid, visually resembling a comet. A LEICA DMLS fluorescence microscope (400 X magnification) was used for slide analysis. For each sample, two slides were prepared. Fifty cells (25 per individual) were randomly scored using a public domain NIH-Image program [11].

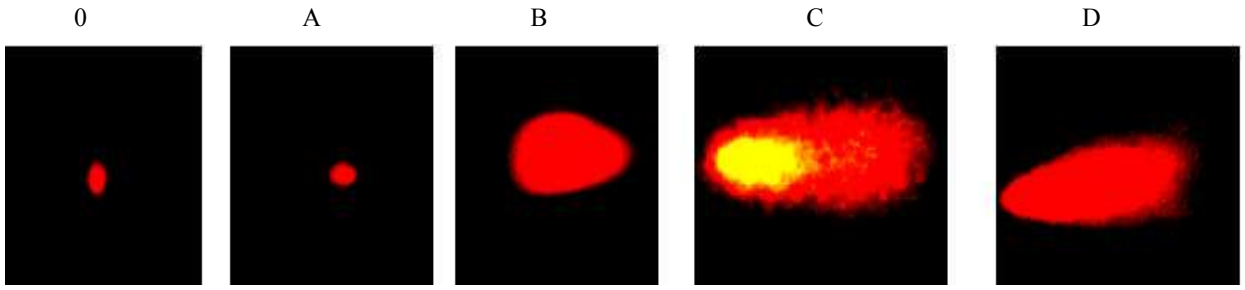

Fig. 1: The different cell damage stages in the comet assay in fish and aquatic insects

Image analysis was mainly made on the increased fluorescence in the tail region, referring to the percentage of DNA in the tail (TD %), the tail length (TL), or the product of both, called the tail moment (TM). TM was the chosen parameter for the comparative analysis between conditions due to its responsiveness. Data on TD and TL were measured (but not shown) in a pre analysis phase because, when using a derived parameter such as TM, the original parameters should be considered as suggested by Koppen [12]. Control comet cells are represented by the nucleoid core only, normally with minimal DNA migration. Any healthy cell typically contains a certain proportion of SBs in its DNA, the result of either spontaneous damage or DNA breakage necessary to DNA functions such as its synthesis [12]. Besides TM, the frequency of apoptotic cells (ACs) was also accessed through the comet assay data. The mean head intensity and area were considered together to better define an AC in this work. An interval of acceptance was defined by the mean ± standard deviation for both comet parameters and values below this limit were highlighted and analyzed in detail. A positive result was considered if both parameters (mean head intensity and area) were demarked and confirmation was made by evaluation of the respective comet image in comparison to the fan-like pattern previously described by Tice *et al*. [5]. These cells were included in the counting of 50 comets per individual but excluded from any TM image analysis or statistic calculations, as they represent dead/dying cells [13]. Each cell was scored visually as belonging to either one of the five specific damage stages based on the relative intensity of the head and tail fluorescence (from undamaged DNA stage 0 to maximal damaged DNA, stage D). Undamaged DNA stage 0 has no tail, damaged DNA stage A has a tail length equal to or shorter than the length of head diameter, damaged DNA stage B has a tail length 1.1-3.5 times longer than the head diameter, damaged cell stage C has a tail length greater than 3.5 times the head diameter, damaged DNA stage D has no “head” since all DNA migrated to the tail [14].

Statistical Analyses: The significance of the differences was evaluated using the one way ANOVA by comparing comet %, tail length and tail moment of samples.

RESULTS AND DISCUSSION

In recent years, there has been an increasing interest in the effects of toxicants on fish health due to the importance of fishing in rivers, ponds and estuaries exposed to wastes of the productive activity. Chemical contamination of water may affect molecular mechanisms of fish and induce genetic alterations that can be used as markers of DNA alterations in environmental pollution [15].

In the present study, the comet assay was utilized as a biomarker of the genotoxic potential in the River Nile, which was found to be effective in showing double the DNA damage in aquatic insects and fish from different aquatic sources.

Comet Assay Analysis: Following electrophoresis the presence of DNA strand breaks allows fragments of DNA to move from the nucleoid core towards the anode, thus resulting in ‘comet’ formation [10]. With increasing amounts of damage more DNA migrates into the tail region and is quantified in terms of either increased fluorescence in the tail region (percentage DNA in the tail or alternatively the percentage of DNA in the head region), tail length, or a product of these two measurements, tail moment (Fig:1 ;B - D). The results of DNA damage stages were represented in Table (1) and Fig. (1). The percentage of cells in each damage stage and the score of damage were used as parameters for measuring genotoxicity. The percentage of cells in each damage stage, the score of damage and the tail length from 50 cells per sample (in triplicate) were used as the parameters for measuring genotoxicity. In comparison to control cells (Fig: 1; 0 and A) consisted of a nucleotid core with zero or minimal DNA migrating into the tail region. DNA damage stages from stage B to D were

recorded in fish and aquatic insects collected from the mixed point of wastewater, while in samples collected from the mixed point of agriculture drain, the damage stages were only from stage A to C. The control samples collected from the main stream of River Nile before branches have minimal DNA damage stages (A-B) Table (2). Similar results were obtained by Avishai *et al*. [14] who used comet assay as an ecotoxicological monitoring tool for evaluation of genotoxicity in the Kishon River, which is the most polluted River in Israel. It was found that most of samples exhibited high rates of severe DNA damage (stage C and D) than the control samples. The results of the present study were confirmed by others used the comet assay as a very sensitive tool for environmental monitoring which can detect a very low level of DNA damage [17, 18].

Tables (2) summarized the results for DNA damage occurred in fish and aquatic insects from different aquatic sites. The percentage of DNA in the tail region (tail % DNA) was considered to be the most appropriate criterion for quantifying DNA damage. The results indicated higher tail moment in fish and aquatic insects collected from the mixed point of wastewater than the mixed point of agriculture drain and the main stream of River Nile before branches. Similar results were obtained by Abd-Allah *et al*. [18] who used the alkaline Comet assay as a simple and rapid method by which DNA damage can be demonstrated as a function of tail moment. Though a previous study revealed that tail length and tail moment can provide similar results [19]. Moreover, the results obtained by Pereira *et al*. [20] were in agreement with the present study in which tail moment proved to be more responsive than tail length. Additionally, TM has the advantage of considering damage expressed as a short tail with a high fraction of DNA or a long tail with a low fraction of highly fragmented DNA [22]. From Table (2) it can be seen that the highest percentage of comet cells were found in fish and aquatic insects collected from the mixed point of wastewater followed by the mixed point of agriculture drain. The main stream of River Nile before branches has the lower comet percentage. The high percentages of comet cells may be due to that the River may be mixed with genotoxic materials from domestic waste and agricultural runoff that contain pesticides and fertilizers. Potent genotoxins usually found in domestic wastes, which are known to be present in human sanitary outflows found in municipal discharges [22]. Van *et al*. [23] demonstrated that the Comet assay in *Tilapia rendalli* should be a good biomarker. The assay is reliable, relatively cheap and easy to perform. The DNA is sensitive to pollutant exposure and effects (DNA breakage) serve as an early warning parameter. Fish biomarker may be useful tools in several steps of the risk assessment process: effect, exposure and hazard assessment, risk characterization or classification and monitoring the environmental quality of aquatic ecosystems.

Table 1: DNA damage stages in fish and aquatic insects collected from different aquatic sites:

Area	Samples	Comet %	Stage A	Stage B	Stage C	Stage D
The mixed point of agriculture drain	Fish	16	2	4	2	0
	Insects	18	2	5	2	0
The mixed point of wastewater	Fish	18	0	3	4	2
	Insects	20	0	3	2	4
The main stream of River Nile before branches	Fish	10	2	4	0	0
	Insects	13	3	3	0	0

Table 2: DNA damage in fish and aquatic insects collected from different aquatic sites:

Area	Number of cells with comet	Samples	Comet %	Tail length	Tail %	Tail moment
the mixed point of agriculture drain	Fish	8	16	1.7	57 %	1
	Insects	9	18	0.9	55.4 %	0.5
The mixed point of wastewater	Fish	9	18	2.4	52.8	1.3
	Insects	10	20	2.65	54.5	1.4
The main stream of River Nile before branches	Fish	5	10	1.13	63.3	0.7
	Insects	6	13	1.17	50	0.5

Results are expressed as means. N= 50 cells (3 replicate slides)

Also it can be seen from Tables (1 and 2) that there is a degree of DNA damage in fish and aquatic samples collected from the main stream of River Nile before branches; which considered as a control site. Previous studies also stated that any healthy cell typically contains a certain proportion of single strands in its DNA, the results of either spontaneous damage or DNA breakage necessary to DNA functions as its synthesis [12]. In this concern, Sasaki *et al*. [24] suggested that the determination of genotoxicity as a result of environmental contamination of water should be conducted with the water as a whole and not specifically for each (contaminating) component and that the comet would be a good test for this type of monitoing. The data on genotoxicity in aquatic insects and fish in different aquatic sources in River Nile demonstrated the poor quality of that environment which may be due to the high sewage discharge, human activities, the movement of water which is more or less stagnant and high electrical conductivity which is often used as an indirect index of pollution [23].

The statistical analysis of DNA damage using one way ANOVA for tail moment of fish and aquatic insects collected from different aquatic sites showed no significant differences between samples collected from control site (P= 0.08). Abd-Allah *et al*. [18] obtained similar results when analyzed values of DNA tail moment of catfish treated with mycotoxins. While when analyzing other comet parameters (comet% and tail length) samples were significantly different (P= 0.04). Best and Ross [25] also obtained similar results by analysis of variance of tail moment revealed significant differences between infected and control fish.

CONCLUSION

The present study confirmed the applicability of the comet assay as a sensitive tool for environmental monitoring. Also it can be suggested that aquatic insects and fish are good bio-indicators of genotoxicity.

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