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First application of comet assay in blood cells of Mediterranean loggerhead sea turtle (*Caretta caretta*)

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ABSTRACT

The aim of this study was to validate the comet assay in erythrocytes of *Caretta caretta*, a species never investigated for genotoxicity. We studied 31 loggerhead sea turtles from three Italian marine rescue centres. Peripheral blood samples were collected from all the animals and the comet assay applied. All comet cells were analysed using two methods: visual scoring and computer image analysis. The % DNA in tail mean value ± SD and Damage Index were 21.56 ± 15.41 and 134.83 ± 94.12, respectively. A strong and statistically significant statistically correlation between the two analytical methods was observed ($r = 0.95$; $p < 0.05$). These results demonstrate that the comet assay is a useful method to detect the possible effects of genotoxic agents in loggerhead sea turtle and to increase the knowledge about the ecotoxicological health status of this threatened species.

1. Introduction

The loss of DNA integrity can cause genotoxic effects, such as DNA base modifications, strand breaks, depurination and cross-linkages (Frenzilli et al., 2004). These types of damages, if not repaired, can initiate a cascade of biological consequences at the level of cells, organs, whole animal and even populations through mutations, cancers, birth defects, reduced growth, abnormal development and the reduced survival of embryos, larvae and adults (Lee and Steinert, 2003).

Several tests have been developed to evaluate DNA damage in living cells, such as the comet assay, diffusion assay, sister chromatid exchanges, alkaline elution, erythrocytic nuclear abnormalities assay and micronucleus test (Frenzilli et al., 2009; Goumenou and Machera, 2004; Oliveira et al., 2010; Wilson and Thompson, 2007).

The comet assay or Single Cell Gel Electrophoresis (SCGE) is a sensitive method used as an indicator of genotoxicity and an effective biomarker for detecting DNA strand breaks, cross-links and alkali-labile sites in aquatic animals (Frenzilli et al., 2009). The advantages of this assay include the relative ease of application to most eukaryotic cell tissue types, its sensitivity for detecting low levels of DNA damage (1 break per 10^{10} Da of DNA), the detection of multiple classes of DNA damage with a small number of cells (~10,000), and the generation of single-cell data (Dhawan et al., 2009).

The comet assay is mainly used to evaluate *in vivo* and *in situ* exposures in freshwater fish species, whereas only few studies have used the comet assay with marine fish (Frenzilli et al., 2009; Jha, 2008). To the best of our knowledge, there are no genotoxicological studies on marine long-living species such as the loggerhead sea turtle.

Loggerhead turtles (*Caretta caretta*) are carnivorous, foraging primarily on benthic invertebrates and fish throughout their distribution range. The high diversity in the type of their prey demonstrates versatility in foraging behaviour, suggesting that the loggerhead is a generalist (Plotkin and Amos, 1990). *C. caretta*, the most common marine turtle species in the Mediterranean basin, is currently classified as "endangered" by the IUCN (International Union for the Conservation of Nature and Natural Resources, the World Conservation Union), and it is also considered "endangered" in the Mediterranean basin (Marine Turtle Specialist Group, 1996).

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Its life history traits characterized by delayed maturity and longevity make it susceptible to numerous anthropogenic threats (Lazar et al., 2011).

The Mediterranean Sea suffers from PAHs (polycyclic aromatic hydrocarbons), OCs (organochlorines) and heavy metal contamination. Urbanization, tourism, wastewaters, the oil industry, maritime traffic, and agriculture, are the main problems threatening the Mediterranean marine ecosystem (EEA Report, 2006). The levels of contaminants in the Mediterranean basin were showed to be higher than other seas and oceans (Aguilar et al., 2002), because of the slow renewal of waters in the Mediterranean system, the large concentrations of human populations along its coasts and the intense industrial development (Maffucci et al., 2005). Anthropogenic contaminants in the Mediterranean Sea can cause genotoxic effect in the DNA of living cells of marine species.

Because of its high risk category for local extinction, or extirpation, plus the contaminated state of the Mediterranean Sea, ecotoxicological studies on the species *C. caretta* are urgently needed in order to better understand its ecotoxicological health status. The aim of the present study was to validate the comet assay in blood cells of Mediterranean loggerhead sea turtles for future application in order to increase our knowledge about the ecotoxicological health status of this threatened species.

2. Material and methods

2.1. Sampling methods

The blood collection procedure was carried out in strict accordance with the relevant national and international guidelines under CITES permits (CITES Nat. IT025IS, Int. CITES IT 007). University of Siena and the museum of "Accademia dei Fisiocritici" (which is the devoted Institution in Siena to release the permits) approved the study. CITES authorizes to collect and carry scientific material undergoing CITES rules.

Between 2008 and 2011, 31 blood samples were obtained from loggerhead sea turtles rescued in three different Italian marine rescue centres (Talamone-Tuscany, Manfredonia-Apulia and Brancaleone-Calabria). The causes of recovery can include entanglement in fishing nets, ingestion of hooks or plastic, traumatic injuries caused by boat strikes, crude oil ingestion, malnutrition, skin diseases and other unidentified causes. Once rescued, all turtles were transported to rescue centres where they were maintained in tanks filled with seawater at controlled temperature and natural light cycles. All turtles were sampled at least one month after the recovery, and only when the animals were in stable condition according to the center's veterinarian. The curve carapace length (CCL), and weight of the turtles were collected. On the basis of CCL (Casale et al., 2009), all turtles were classified in three age classes (Table 1). Because they were too young, the sex could not be determined in many of the individual turtles and so was not included in statistical analyses. Two mL of blood were collected from the cervical sinus of each animal using a disposable syringe with the support of the facility veterinarian and immediately stored at 4 °C until processed. All samples were analyzed within 24 h.

Table 1
Age classes, number and CCL (curve carapax length) range of *Caretta caretta* specimens. The age classes were determined on the von Bertalanffy growth curve as reported by Casale et al. (2009).

Class	*N*	CCL range (cm)
1	8	≤39.9
2	13	40–58.4
3	10	58.5–79

2.2. Comet assay

Cell viability was determined before conducting the comet assay, using the trypan blue dye exclusion technique. Since there was no previous report of the application of the comet assay in *C. caretta*, we had to carry out a preliminary study using the protocol previously established for fish erythrocytes (Frenzilli et al., 1999, modified), testing critical parameters such as cell suspension, unwinding and electrophoresis conditions. Based on our previous studies (unpublished), we used H_2O_2 (30 μM) as a positive control and unwinding and electrophoresis conditions as reported below.

Blood was diluted in PBS (1:100), embedded in agarose (0.5% low-melting agarose) and layered on two slides per sample, predipped in 1% normal melting agarose. The slides were immersed into a freshly made lysis solution (2.5 M NaCl, 10 mM Tris, 0.1 M EDTA, 1% Triton X-100, and 10% DMSO, pH 10) for at least 1 h at 4 °C in the dark. The slides were then placed on a horizontal electrophoresis tray previously filled with freshly prepared cold alkaline buffer and left for 10 min to allow DNA unwinding. Electrophoresis was performed at 25 V and 300 mA for 10 min. Slides were then neutralized (3 × 5 min; 0.4 M Tris, pH 7.5) and stained with SYBR® Safe 1:10.000 in TE (10 mM Tris–HCl pH 7.5 and 500 mM EDTA pH 7.5) buffer. A total of 100 cells per sample (50 from each duplicate sample) were examined under the epifluorescence microscope (Olympus BX41) at 400× magnification (Fig. 1). Images were analysed in parallel using two methods: visual score method (Collins, 2004) and computer image analysis (Burlinson et al., 2007).

The visual method was based on the cells being scored visually, and ranked into one of five classes according to tail size and shape (from undamaged, class 0, to maximally damage, class 4). According to the tail extension in relation to the head diameter, the comets are classified as class 0 (without tail), class 1 (with tail extension smaller than the head diameter), class 2 (with tail extension between 1 and 2 times bigger than the head diameter), class 3 (with tail extension 2 times bigger than the head diameter) and class 4 (practically without head but with a large tail). Comets were individually scored (Damage Index, DI) according to class and DI ranged from 0 (completely undamaged: 100 cells × 0) to 400 (maximum damage: 100 cells × 4) (Collins, 2004).

The computer image analysis quantified the amount of DNA damage as the percentage of DNA in tail (% DNA in tail) using an image analyser (Komet 6.0 Software, Kinetic Imaging Ltd.).

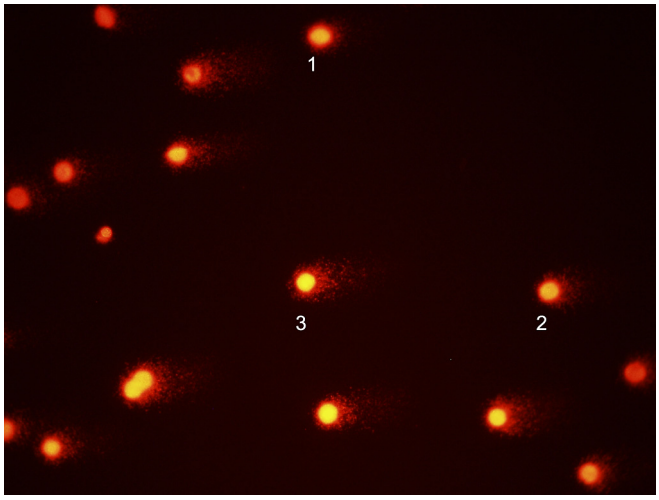

Fig. 1. SYBR® Safe-stained images (400× magnification) of erythrocytes of *Caretta caretta* subjected to single cell gel electrophoresis. Numbers indicates classes of some nucleoids according to the visual score method.