Estimates of DNA strand breakage in bottlenose dolphin (*Tursiops truncatus*) leukocytes measured with the Comet and DNA diffusion assays

Adriana Díaz1, Sandra Carro1, Livia Santiago1, Juan Estévez1, Celia Guevara2, Miriam Blanco2, Laima Sánchez2, Liena Sánchez2, Nirka López2, Danilo Cruz2, Ronar López2, Elizabeth B. Cuetara3 and Jorge Luis Fuentes4

1Departamento de Radiobiología, Centro de Aplicaciones Tecnológicas y Desarrollo Nuclear, C. Habana, Cuba.
2Departamento de Salud Animal, Acuario Nacional de Cuba, C. Habana, Cuba.
3Laboratorio de Farmacología Clínica y Experimental, Instituto Nacional de Oncología y Radiobiología, C. Habana, Cuba.
4Laboratorio de Microbiología y Mutagénesis Ambiental, Escuela de Biología, Facultad de Ciencias, Universidad Industrial de Santander, Bucaramanga, Colombia.

Abstract

The analysis of DNA damage by mean of Comet or single cell gel electrophoresis (SCGE) assay has been commonly used to assess genotoxic impact in aquatic animals being able to detect exposure to low concentrations of contaminants in a wide range of species. The aims of this work were 1) to evaluate the usefulness of the Comet to detect DNA strand breakage in dolphin leukocytes, 2) to use the DNA diffusion assay to determine the amount of DNA strand breakage associated with apoptosis or necrosis, and 3) to determine the proportion of DNA strand breakage that was unrelated to apoptosis and necrosis. Significant intra-individual variation was observed in all of the estimates of DNA damage. DNA strand breakage was overestimated because a considerable amount (~29%) of the DNA damage was derived from apoptosis and necrosis. The remaining DNA damage in dolphin leukocytes was caused by factors unrelated to apoptosis and necrosis. These results indicate that the DNA diffusion assay is a complementary tool that can be used together with the Comet assay to assess DNA damage in bottlenose dolphins.

Key words: Comet assay, DNA diffusion assay, DNA strand breakage, *Tursiops truncatus*.

Received: May 30, 2008; Accepted: October 10, 2008.

Introduction

The single cell gel electrophoresis (SCGE) or Comet assay, as introduced by Singh *et al.* (1988), is a technique that detects DNA strand breakage and alkali labile sites by measuring the migration of DNA from immobilized individual cell nuclei. In this assay, cells are embedded in agarose gel on microscopic slides and lysed and electrophoresed under alkaline condition. Cells with damaged DNA show increased migration of DNA fragments from the nucleus. The length of the migration indicates the amount of DNA breakage, and the DNA damage can be estimated by both manual microscopic and computerized image scoring analyses (Olive *et al.*, 1990; McKelvey-Martin *et al.*, 1993; Fairbairn *et al.*, 1995; Kobayashi *et al.*, 1995). The minimal technical requirements for conducting this assay in human cells *in vitro* and *in vivo* have been well established (Tice *et al.*, 2000; Hartmann *et al.*, 2003).

The widespread use of the Comet assay in biomonitoring studies of aquatic organisms is related mainly to its simplicity, low cost and greater sensitivity to xenobiotics when compared with other techniques (Mitchelmore and Chipman, 1998; Cotelle and Ferard, 1999; Lee and Steinert, 2003; Frenzilli *et al.*, 2009). The Comet assay has been used to detect DNA damage induced by hydrogen peroxide and methyl mercury in bottlenose dolphin (*Tursiops truncatus*) lymphocytes *in vitro* (Betti and Nigro, 1996; Taddei *et al.*, 2001), and by hydrogen peroxide and benzo[a]pyrene-7,8-dihydro-diol-9,10-epoxide in sea lion lymphocytes (El-Zein *et al.*, 2006). These results suggest that this assay may be a sensitive tool for monitoring DNA damage in marine mammals.

However, the use of the Comet assay in biomonitoring studies has been questioned because positive results in this assay do not necessarily reflect genotoxicity but may arise from DNA damage, *i.e.*, double-strand breakage, associated with apoptosis and necrosis (Olive *et al.*, 1993; Olive and Banath, 1995; Steinert, 1996; Godard *et al.*, 1999;
To overcome this limitation, the Comet assay may be used in combination with related methodologies that estimate DNA fragmentation associated with apoptosis and necrosis, such as the DNA diffusion assay (Singh, 2000a,b). In the latter assay, small molecular weight DNA fragments generated during apoptosis and necrosis diffuse in the agarose matrix to give an apparent nuclear diameter that is ~3 times greater than the mean nuclear size as a consequence of the high dispersion of DNA. Based on the structural differences between apoptotic and necrotic nuclei, Singh (2000a,b) recommended that the DNA diffusion assay be used to distinguish apoptotic from necrotic cells. However, other investigators have been unable to differentiate between apoptotic and necrotic cells when using this assay (Gichner et al., 2005). The use of the DNA diffusion assay to assess apoptosis has been reviewed by Singh (2005) and its application in a small number of environmental studies has yielded promising results (Nigro et al., 2002; Frenzilli et al., 2004; Del Barga et al., 2006).

We have initiated a long-term monitoring project aimed at evaluating the life quality of bottlenose dolphins living in semi-captive and captive conditions. In view of the potential use of the Comet assay in the biomonitoring of dolphins and that a concurrent assessment of apoptosis, necrosis and genotoxin-induced DNA strand breakage is critical for the correction interpretation of this assay, the aims of this work were: 1) to evaluate the usefulness of the Comet or single cell gel electrophoresis (SCGE) assay to detect DNA strand breakage in dolphin leukocytes, 2) to use the DNA diffusion assay to determine the amount of DNA strand breakage associated with apoptosis and necrosis, and 3) to determine the proportion of DNA strand breakage that was unrelated to apoptosis and necrosis.

### Materials and Methods

#### Capture and living conditions of the dolphins

Twenty-five bottlenose dolphins (Table 1) were captured in the Sabana-Camagüey archipelago on the northern coast of Cuba and their sex was determined visually. Age was estimated based on the dolphins size, teething state and body marks at the time of capture. The dolphins were initially quarantined in sea-hoops located close to the capture

| N. | Dolphin name | Capture date | Origin         | Sex¹ | Length¹ (m) | Age group² |
|---|--------------|--------------|----------------|------|-------------|------------|
| 1 | Ciceron      | 11/08/2000   | Aguada key     | M    | 2.13        | Juvenile   |
| 2 | Serena       | 12/08/2000   | Aguada key     | F    | 2.25        | Adult      |
| 3 | Salome       | 14/08/2000   | Horseshoe key  | F    | 2.19        | Juvenile   |
| 4 | Jade         | 04/12/2001   | San Juan Point | F    | 2.08        | Juvenile   |
| 5 | Javy         | 04/12/2001   | San Juan Point | F    | 1.98        | Calf       |
| 6 | Lía          | 15/07/2002   | Santa Maria key| F    | 1.81        | Calf       |
| 7 | Lili         | 28/08/2002   | Glad Point     | F    | 2.04        | Juvenile   |
| 8 | Xena         | 25/10/2002   | Caibarien bay  | F    | 2.18        | Juvenile   |
| 9 | Maria        | 18/06/2003   | Santa Maria key| F    | 2.49        | Adult      |
| 10 | Mara         | 18/06/2003   | Santa Maria key| F    | 2.01        | Juvenile   |
| 11 | Merlin       | 18/06/2003   | Santa Maria key| M    | 2.12        | Juvenile   |
| 12 | Maida        | 22/06/2003   | Glad Point     | F    | 2.40        | Adult      |
| 13 | Mihai        | 22/06/2003   | Glad Point     | M    | 1.86        | Calf       |
| 14 | Musa         | 22/06/2003   | Glad Point     | F    | 1.94        | Calf       |
| 15 | Marcelo      | 29/07/2003   | Guarana key    | M    | 2.40        | Adult      |
| 16 | Malú         | 08/08/2003   | Horseshoe key  | F    | 2.33        | Adult      |
| 17 | Monica       | 08/08/2003   | Horseshoe key  | F    | 2.28        | Adult      |
| 18 | Margarita    | 09/08/2003   | Guarana key    | F    | 2.07        | Juvenile   |
| 19 | Montse       | 02/09/2003   | Guarana key    | F    | 2.00        | Juvenile   |
| 20 | Melany       | 03/09/2003   | Drunk key      | F    | 2.28        | Adult      |
| 21 | Milo         | 08/09/2003   | Guarana key    | F    | 2.28        | Adult      |
| 22 | Moon         | 08/09/2003   | Guarana key    | F    | 2.19        | Juvenile   |
| 23 | Maja         | 08/09/2003   | Guarana key    | F    | 1.93        | Calf       |
| 24 | Mégame       | 18/10/2003   | Guarana key    | M    | 2.17        | Juvenile   |
| 25 | Milano       | 18/10/2003   | Guarana key    | M    | 2.11        | Juvenile   |

¹Determined at the time of capture. ²Age categories were: calf < 2.0 m long, juvenile 2.00-2.20 m long and adult > 2.21 m long.
site where they were examined by a veterinarian and under-went blood and spiracle analyses. Only visually healthy and asymptomatic dolphins that willingly consumed frozen fish were transferred to the dolphinarium at the Cuban National Aquarium (CNA). The dolphinarium was supplied with water from a subterranean well via a semi-closed feeding system with filters and a water renewal rate of 10% per hour. This system was permanently and automatically supplemented with sodium hypochlorite (final concentration of free chloride: 0.3 mg/L). The quality of water used in the dolphinarium was based on the parameters established by the Cuban guidelines for fishery zones (NC25:1999).

Blood sampling

Blood samples were obtained by vacuum using sterile, heparinized tubes and were always shipped and stored on ice until assayed.

Analysis of serum genotoxicity

Serum genotoxicity was measured indirectly using the SOS Chromotest (Quillardet et al., 1982) with modifications. Escherichia coli PQ37 cells were grown to an OD_{600nm} of 0.4 in Luria-Bertani (LB) media supplemented with ampicillin (50 μg/mL) at 37 °C, with shaking (100 rpm). Exponential phase cultures were diluted ten-fold in fresh 2X LB media supplemented with ampicillin (100 μg/mL) and then dispensed in Eppendorf tubes (250 μL per tube) containing 225 μL of serum. When metabolic activation was required, 25 μL of phenobarbital/5,6 benzo[α]pyrene-induced rat liver S9 fraction from Moltox was used (final concentration in the activation mixture: 0.4%, v/v). In experiments without metabolic activation, the rat liver S9 fraction was substituted with sterile distilled water. The reference mutagens 2-acetylaminofluorene (2-AF) (100 μg/mL) and γ-rays (150 Gy delivered by a Co^{60} PX-γ-30M Russian irradiator at a dose rate of 33-42 Gy/min) were used as positive controls in experiments with and without metabolic activation, respectively. The cells were exposed to serum samples for 30 min at 8 °C and then cultured for 2 h at 37 °C. β-galactosidase and alkaline phosphatase activities were assayed as described by Fuentes et al., (2006). The criterion for genotoxicity was the SOS induction factor (SOSIF), as defined by Quillardet et al., (1989): SOSIF = [β-galactosidase/alkaline phosphatase]_treatment/[β-galactosidase/alkaline phosphatase]_negative control. Serum samples were classified as not genotoxic (SOSIF < 1.5), inconclusive (SOSIF = 1.5-2.0) or genotoxic (SOSIF > 2.0) (Kevekordes et al., 1999).

Estimation of DNA strand breakage in dolphin leukocytes

DNA strand breakage in dolphin leukocytes was estimated by using the Comet assay, as described by Singh et al., (1988), with modifications to the silver staining as indicated by Garcia et al., (2004). The DNA damage was scored (see Figure 1) based on five categories (0-4), as indicated by Collins et al., (1997). The total amount of DNA strand breakage was expressed in total arbitrary units (AU) as follows: AU_{T} = N_{0} x 0 + N_{1} x 1 + N_{2} x 2 + N_{3} x 3 + N_{4} x 4, where N_{i} is the number of nuclei scored in each category (Collins, 2002). One hundred cells per slide and two slides per blood sample were analyzed and the results of at least two independent experiments were averaged to obtain the AU_{T} for each dolphin.

Since positive Comet results do not necessarily reflect genotoxicity because DNA strand breakage may be associated with cellular apoptosis and necrosis, we used the DNA diffusion assay to determine the percentage of apoptotic/necrotic cells in each blood sample. For this assay, the cells were processed in a manner similar to the Comet assay, except that the nuclei were not subjected to electrophoresis. Nuclei with a diameter > 3 times the mean nuclear diameter were considered apoptotic/necrotic (Nigro et al., 2002). The total number of nuclei (minimum of 100 cells per slide) and the number of apoptotic/necrotic nuclei in each field were counted and the latter then expressed as a percentage of the former. As in the Comet assay, two slides per blood sample were analyzed and the results of at least two independent experiments were averaged to obtain the percentage of apoptotic/necrotic nuclei for each dolphin.

Based on the total number of DNA strand breakages (AU_{T}) estimated with the Comet assay and the percentage of apoptotic/necrotic nuclei (%N_{apoptotic/necrotic}) for each dolphin, the proportion of remaining DNA strand breakages was calculated (in arbitrary units) as:

\[
AU_{R} = AU_{T} - \frac{\%N_{apoptotic/necrotic} \times AU_{T}}{100}
\]

where AU_{R} corresponds to non-apoptotic/necrotic DNA strand breaks.

Statistical analysis

The results were expressed as the mean ± S.E.M., where appropriate. In all cases, the data passed the Kolmogorov-Smirnov and F-maximum tests for normality and variance homogeneity, respectively, so that parametric tests

Figure 1 - A. Bottlenose dolphin leukocytes analyzed by the Comet assay. Nuclei from undamaged leukocytes consisted of a head (nuclear core) with little or no DNA migrating into the tail region. Nuclei from damaged leukocytes consisted of a head with DNA migrating into the tail region as a result of strand breakage. B. Bottlenose dolphin leukocytes analyzed by the DNA diffusion assay. Nuclei that diffused through the agarose gel were considered as apoptotic/necrotic nuclei.
were used to analyze the data. When a significant F-value was obtained in one-way analysis of variance (ANOVA) the groups were subsequently compared with Students t-test. Product-moment (Pearson) correlation analysis was used to examine the relationship between estimates of DNA damage (AU_T, \%N_{apoptotic/necrotic} and AU_R). A value of p < 0.05 indicated significance. All statistical analyses were done with STATISTICA V.6 software (StatSoft Inc).

Results

Table 2 shows the total DNA strand breakage in dolphin leukocytes, expressed as total arbitrary units (AU_T) of the Comet assay. There was significant inter-individual variation (p < 0.05 in ANOVA) in the proportion of DNA strand breakage between the dolphins Jade (93 ± 48) and Mara (305 ± 28) (mean: 191 ± 34). Table 2 also shows the percentage of apoptotic/necrotic nuclei (%N_{apoptotic/necrotic} for each dolphin; there was significant inter-individual variation (p < 0.05 in ANOVA) between the dolphins Moon (6 ± 0) and Maja (77 ± 0) (mean: 29 ± 4).

The proportion of DNA strand breakage (AU_R) not attributable to apoptosis and necrosis was calculated based on the total DNA strand breakage (AU_T) estimated with the Comet assay and the percentages of apoptotic/necrotic nuclei (%N_{apoptotic/necrotic}). The AU_R ranged from 34 ± 1 for Maja to 238 ± 33 for Mihai (mean: 125 ± 12). AU_R estimates, but not the AU_T, were significantly higher (t-value = -2.16, p < 0.03) in males than females (164 ± 30 vs. 119 ± 14, respectively), suggesting that AU_R is a better indicator than AU_T for detecting DNA strand breakage in bottlenose dolphins. This finding contrasts with the results reported for beluga whales using chromosomal aberration and sister chromatid exchange assays (Gauthier et al., 1999), but is not surprising since chromosomal aberrations occur on a much different scale than strand breakage and may be attributed to different factors. Both of the estimates of DNA damage (AU_T and AU_R) increased significantly with dolphin age (data not shown), in agreement with previous findings obtained using the micronucleus assay (Zamora-Perez et al., 2006).

To determine whether the residual (non-apoptotic/necrotic) DNA strand breakage (AU_R) originated from exposure to genotoxic compounds we examined the genotoxicity of dolphin serum using the SOS chromotest (Quillardet et al., 1982). This test detects a wide range of genotoxic compounds, including marine contaminants such as polycyclic aromatic hydrocarbons and organochlorine compounds (Quillardet and Hofnung, 1993). In experiments with metabolic activation, the SOSIF values ranged from 0.2 ± 0.0 to 0.8 ± 0.0 (mean: 0.5 ± 0.2) while in experiments without metabolic activation this indicator ranged from 0.3 ± 0.0 to 0.9 ± 0.6 (mean: 0.6 ± 0.4) (Table 2). These data indicated that there were no genotoxic compounds in dolphin blood.

| Table 2 - Leukocyte DNA damage and serum genotoxicity in bottlenose dolphins. |
|-----------------------------|-----------------------------|-----------------------------|
|                            | Leukocyte DNA damage        | Serum genotoxicity          |
|                            | AU_T                        | %NuclApMon                 | SOSIF (S_0 +) | SOSIF (S_-) |
| Dolphin name               | AU_R                        |                            |              |
| Ciceron                    | 257 ± 27                    | 55 ± 14                    | 117 ± 41     | 0.5 ± 0.0    | 0.7 ± 0.2    |
| Serena                     | 263 ± 93                    | 26 ± 5                     | 196 ± 72     | 0.4 ± 0.1    | 0.8 ± 0.1    |
| Salome                     | 242 ± 118                   | 30 ± 8                     | 166 ± 58     | 0.5 ± 0.0    | 0.7 ± 0.2    |
| Jade                       | 93 ± 48                     | 26 ± 3                     | 68 ± 33      | 0.4 ± 0.0    | 0.9 ± 0.3    |
| Javy                       | 154 ± 61                    | 22 ± 2                     | 120 ± 47     | 0.5 ± 0.2    | 0.7 ± 0.2    |
| Lia                        | 161 ± 73                    | 28 ± 18                    | 110 ± 43     | 0.6 ± 0.1    | 0.8 ± 0.3    |
| Lili                       | 160 ± 57                    | 24 ± 5                     | 118 ± 37     | 0.4 ± 0.1    | 0.5 ± 0.2    |
| Xena                       | 149 ± 56                    | 29 ± 6                     | 100 ± 29     | 0.6 ± 0.2    | 0.7 ± 0.1    |
| Maria                      | 186 ± 43                    | 28 ± 16                    | 131 ± 18     | 0.6 ± 0.0    | 0.4 ± 0.0    |
| Mara                       | 305 ± 28                    | 64 ± 0                     | 110 ± 10     | 0.2 ± 0.0    | 0.4 ± 0.0    |
| Merkel                     | 202 ± 62                    | 29 ± 0                     | 143 ± 44     | 0.8 ± 0.0    | 0.4 ± 0.0    |
| Maida                      | 102† 1                      | 46†                        | 55†          | 0.8†         | 0.8†         |
| Mihai                      | 298 ± 42                    | 20 ± 0                     | 238 ± 33     | 0.3 ± 0.0    | 0.5 ± 0.0    |
| Musa                       | 151† 1                      | 28†                        | 109†         | 0.3†         | 0.6†         |
| Marcelo                    | 205 ± 5                     | ND                         | ND           | 0.5 ± 0.0    | 0.4 ± 0.0    |
| Malù                       | 240 ± 22                    | 50 ± 0                     | 120 ± 11     | 0.6 ± 0.0    | 0.6 ± 0.0    |
| Monica                     | 241† 1                      | 48†                        | 125†         | 0.8†         | 0.4†         |
| Margarita                  | 239 ± 25                    | 73 ± 0                     | 65 ± 7       | 0.6 ± 0.0    | 0.5 ± 0.0    |
| Montse                     | 235 ± 47                    | 20 ± 0                     | 188 ± 37     | 0.2 ± 0.0    | 0.3 ± 0.0    |
| Melany                     | 180 ± 17                    | 13 ± 0                     | 157 ± 10     | 0.4 ± 0.0    | 0.5 ± 0.1    |
| Milo                       | 147 ± 75                    | 23 ± 0                     | 113 ± 58     | 0.4 ± 0.0    | 0.6 ± 0.1    |
| Moon                       | 217 ± 38                    | 6 ± 0                      | 204 ± 36     | 0.5 ± 0.0    | 0.4 ± 0.0    |
| Maja                       | 147 ± 7                     | 77 ± 0                     | 34 ± 1       | 0.3 ± 0.0    | 0.3 ± 0.0    |
| Mégano                     | 165 ± 119                   | ND                         | ND           | 0.3 ± 0.0    | 0.4 ± 0.0    |
| Milano                     | 206 ± 31                    | 10 ± 0                     | 186 ± 28     | 0.5 ± 0.1    | 0.5 ± 0.1    |
| Mean                       | 191 ± 34                    | 29 ± 4                     | 125 ± 12     | 0.5 ± 0.1    | 0.6 ± 0.2    |

The values are the mean ± S.E.M., where appropriate. AU corresponds to arbitrary units, where AU_T is the total DNA damage as measured with the Comet assay, AU_R is the remaining non-apoptotic/necrotic DNA damage and %N_{ApMon} is the percentage of apoptotic/necrotic nuclei. (*) Only one measurement was done. ND = Not determined. SOSIF = SOS induction factor in Escherichia coli PQ37 cells. The SOSIF values for the reference mutagens were 10.6 ± 2.2 for 500 μg of 2-acetylaminofluorene/mL (with metabolic activation) and 9.4 ± 0.4 for 150 Gy of γ-rays (without metabolic activation). The SOSIF for distilled water was 1.0 ± 0.0.

Discussion

In this work, we combined the Comet and DNA diffusivity assays to measure DNA strand breakage in peripheral blood leukocytes of bottlenose dolphins. Nearly a third (~29%) of the DNA strand breaks arose from apoptotic/necrotic events and led to overestimation of DNA cleavage by the Comet assay, as also previously observed for mussels (Steinert, 1996), humans (Tice et al., 2000) and sea...
lions (El-Zein et al., 2006). The total DNA strand breakage (AU<sub>1</sub>) in dolphin leukocytes was 183 ± 17 AU, indicating a moderate level of DNA cleavage. However, as indicated above, this value was overestimated because of the influence of apoptotic/necrotic events. We therefore computed the value for residual (non-apoptotic/necrotic) DNA strand breakage (AU<sub>0</sub> = 125 ± 12) and found this to be negatively correlated (r = -0.31, p < 0.05) with the percentage of apoptotic/necrotic nuclei. Hence, this parameter may provide a better estimate of non-apoptotic/necrotic DNA strand breakage. The mean AU<sub>0</sub> corresponded to ~23% of DNA in the tail and was slightly higher than the limit for non-damaged nuclei (20% of DNA in the tail), as indicated by Collins et al. (1997).

Several studies have shown that marine contaminants such as heavy metals may induce apoptosis (Steinert, 1996; Shenker et al., 2000; Waalkes et al., 2000). We have investigated the involvement of Fe, Cu and Zn in DNA strand breakage induced by apoptotic/necrotic events and found a weak but significant correlation between serum copper levels and apoptotic/necrotic DNA strand breakage (data not shown). Thus, although the copper content of the dolphinarium water and dolphin serum was consistently low, the apoptosis observed here may have been induced by copper ions; no such relationship was observed for iron or zinc. Other environmental contaminants such as organochlorines and polycyclic aromatic hydrocarbons may also induce apoptosis (Salas and Burchiel, 1998; Shin et al., 2000; Frenzilli et al., 2004), but we have not detected genotoxic activity in dolphin serum using the SOS chromotest. Future studies measuring copper and other genotoxin levels in the environments where the dolphins used in this study normally live should improve our knowledge of the importance of this metal in causing apoptosis-related DNA damage.

The AU<sub>0</sub> values clearly indicated that factors other than apoptosis/necrosis affect the integrity of dolphin leukocyte DNA. Based on the negative SOS chromotest results (Table 2) and the classification for DNA strand breakage in which a score of 0-100 indicated no DNA breakage or damage, 101-200 indicated little DNA damage, 201-300 indicated moderate DNA damage, and 301-400 indicated severe DNA damage, we expected baseline AU<sub>0</sub> values of 0-100 AU, i.e., no DNA damage (Kobayashi et al., 1995; Collins et al., 1997). However, nearly 78% of the dolphins had higher than expected AU<sub>0</sub> values. Using the Comet assay, Taddei et al. (2001) estimated that DNA strand breakage in the nuclei of undamaged lymphocytes from bottlenose dolphins resulted in 16%-20% of their total DNA in the tail, which corresponded to 80-100 AU (Collins et al., 1997). These studies suggest that there may be differences in the baseline estimates of DNA strand breakage obtained in vitro and in vivo, and that additional factors that affect the estimates of DNA cleavage must be considered during risk assessment studies in vivo. Variation in the extent of DNA strand breakage in vivo may reflect physiological conditions, such as a transient increase in oxidative stress caused by the diet or a sub-clinical infection (Collins et al., 1997). An understanding of the influence of such factors on DNA cleavage in bottlenose dolphins should improve our estimates of baseline values for DNA strand breakage measured with the Comet assay.

Conclusions

To our knowledge, this is the first estimate of DNA strand breakage obtained with the Comet assay in peripheral blood leukocytes of bottlenose dolphins. Our results indicate that this assay is sufficiently sensitive for assessing the influence of genotoxic substances in bottlenose dolphins. However, the Comet assay overestimates the extent of DNA strand breakage in these cells because of DNA cleavage caused by apoptotic and necrosis. In addition to apoptosis and necrosis, factors other than exposure to genotoxins may also affect the intactness of bottlenose dolphin DNA. Finally, our results indicate that the DNA diffusion assay is a suitable complementary tool for use alongside the Comet assay during risk assessment studies in bottlenose dolphins.

Acknowledgments

This work was done at CEADEN, Cuba and was supported by a grant (PRMA-2059) from the Cuban Ministry of Science, Technology and Environment.

References

Betti C and Nigro M (1996) The Comet assay for the evaluation of the genetic hazard of pollutants in cetaceans: Preliminary results on the genotoxic effects of methyl-mercury on the bottle-nosed dolphin (Tursiops truncatus) lymphocytes in vitro. Mar Pollut Bull 32:545-548.

Collins AR (2002) The comet assay, principles, applications and limitations. In: Didenko VV (ed) In Situ Detection of DNA Damage. Methods and Protocols. V. 203. Humana Press Inc., Totowa, pp 163-177.

Collins A, Dusinska A, Franklin M, Somorovska M, Petrovska H, Duthie S, Fillion L, Panayoitidis M, Raslova K and Vaughan N (1997) Comet assay in human biomonitoring studies: Reliability, validation and applications. Environ Mol Mutagen 30:139-146.

Cotelle S and Férard JF (1999) Comet assay in genetic ecotoxicology: A review. Environ Mol Mutagen 34:246-255.

Del Barga I, Frenzilli G, Scarcelli V, Nigro M, Malmvårn A, Asplund L, Förlin L, and Vaughan N (1999) Comet assay in human biomonitoring studies: Reliability, validation and applications. Environ Mol Mutagen 30:139-146.

El-Zein RA, Hastings-Smith DA, Ammenheuser MM, Trinen-Moslen M, Gulland FM and Ward Jr JB (2006) Evaluation of two different biomarkers for use in the assessment of toxic chemical exposure in California sea lions (Zalophus californianus). Mar Pollut Bull 52:104-120.

Fairbairn DW, Olive PL and O’Neill KL (1995) The comet assay: A comprehensive review. Mutat Res 339:37-59.
Frenzilli G, Scarcelli V, Del Barga I, Nigro M, Förlin L, Bolognese C and Surve J (2004) DNA damage in eelpout (Zoarces viviparus) from Göteborg harbour. Mutat Res 552:187-195.

Frenzilli G, Nigro M and Lyons BP (2009) The comet assay for the evaluation of genotoxic impact in aquatic environments. Mutat Res 681:80-92.

Fuentes JL, Vernehe M, Cuetara EB, Sánchez-Lamar A, Santana JL and Llagostera M (2006) Tannins from barks of Pinus caribbeae Morelet protect Escherichia coli cells against DNA damage induced by γ-rays. Fitterrator 77:116-120.

García O, Mandina T, Lamadrid AI, Díaz A, Remigio A, González Y, Piñeto Y, Rodríguez JE and Alvarez A (2004) Sensitivity and variability of visual scoring in the comet assay. Result of an inter-laboratory scoring exercise with the use of silver staining. Mutat Res 556:23-34.

Gauthier JM, Dubeau H, Rassart É, Jarman WM and Wells RS (1995) A comparison between manual microscopic analysis and computerized image analysis in the single cell gel electrophoresis assay. Mutagenesis 10:155-161.

Hartmann A, Agurell E, Beevers C, Brendler-Schawaab S, Burghaus CM, Spielberg al., Mersch-Sunderman SV, Burghaus CM, Spielberg al. (2003) Recommendations for conducting the in vivo alkaline comet assay. Mutagenesis 18:45-51.

Kevecordes S, Mersch-Sunderman SV, Burghaus CM, Spielberg J, Schmeiser HH, Artl VM and Dunkeberg H (1999) SOS in vivo and in vitro and computerized image analysis in the single cell gel electrophoresis assay. MMS Commun 3:103-115.

Lee RF and Steinert S (2003) Use of the single cell gel electrophoresis/comet assay for detecting DNA damage in aquatic (marine and freshwater) animals. Mutat Res 544:63-64.

McKelvey-Martin VJ, Green MHL, Schmeiser HH, Artl VM and Danenberg K (1999) SOS in vivo and in vitro and computerized image analysis in the single cell gel electrophoresis assay. A European review. Mutat Res 288:47-63.

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

NC:25 (1999) Norma Cubana: Sistema de Normas para la Protección del Medio Ambiente (Hidrosfera). Especificaciones y Procedimientos para la Evaluación de los Objetos Hídricos de uso Pesquero. Oficina Nacional de Normalización, La Habana, 9 pp.

Nigro M, Frenzilli G, Scarcelli V, Gorbi S and Regoli F (2002) Induction of DNA strand breakage and apoptosis in the eel Anguilla anguilla. Mar Environ Res 54:517-520.

Olive PL and Banath JP (1995) Sizing highly fragmented DNA in individual apoptotic cells using the comet assay and a DNA crosslinking agent. Exp Cell Res 221:19-26.

Olive PL, Banath JP and Durand RE (1990) Heterogeneity in radiation-induced DNA damage and repair in tumor and normal cells using the comet assay. Radiat Res 122:86-94.

Olive PL, Frazer G and Banath JP (1993) Radiation-induced apoptosis measured in TK6 human B Lymphoblast cells using the comet assay. Radiat Res 136:130-136.

Quilllardet P and Hofnung M (1993) The SOS Chromotest. Mutat Res 279:235-279.

Quilllardet P, Huisman O, D’Ari R and Hofnung M (1982) SOS Chromotest, a direct assay of induction of an SOS function in Escherichia coli K-12 to measure genotoxicity. Proc Natl Acad Sci USA 79:5971-5975.

Quilllardet P, Frelat G, Nguyen VD and Hofnung M (1989). Detection of ionizing radiations with the SOS Chromotest, a bacterial short-term test for genotoxic agents. Mutat Res 216:251-257.

Salas VM and Burchiel SW (1998) Apoptosis in Daudi human B cell in response to benzo[a]pyrene and benzo[a]pyrene-7,8-dihydriodiol. Toxicol Appl Pharmacol 151:367-376.

Shenker BJ, Guo TL and Shapiro IM (2000) Mercury-induced apoptosis in human lymphoid cells: Evidence that apoptosis pathway is mercurial species dependent. Environ Res 84:89-99.

Shin KJ, Bae SS, Hwang YA, Seo JK, Ryu SH and Suh PG (2000) 2,2’,4,6,6’-pentachlorobiphenyl induces apoptosis in human monocyes cells. Toxicol Appl Pharmacol 169:1-7.

Singh NP (2000a) A simple method for accurate estimation of apoptotic cells. Exp Cell Res 256:328-337.

Singh NP (2000b) Microgels for estimation of DNA strand breaks, DNA protein crosslinks and apoptosis. Mutat Res 455:111-127.

Singh NP (2005) Apoptosis assessment by DNA diffusion assay. Meth Mol Med 11:55-67.

Singh NP, McCoy MC, Tice R and Schnider EL (1988) A simple technique for quantification of low levels of DNA damage in individual cells. Exp Cell Res 175:184-191.

Steinert SA (1996) Contribution of apoptosis to observed DNA damage in mussel cells. Mar Environ 42:253-259.

Taddei F, Scarcelli V, Frenzilli G and Nigro M (2001) Genotoxic hazard of pollutants in cetaceans: DNA damage and repair evaluated in the bottlenose dolphin (Tursiops truncatus) by the Comet assay. Mar Pollut Bull 42:324-328.

Tice RR, Agurell E, Anderson D, Burlinson B, Hartmann A, Kobayashi H, Miyamae Y, Rojas E, Ryu JC and Sasaki YF (2000) Single cell gel/comet assay: Guidelines for in vitro and in vivo genetic toxicology testing. Environ Mol Mutagen 35:206-221.

Waalkes MP, Fox DA, States JC, Patierno SR and McCabe Jr MJ (2000) Metals and disorders of cell accumulation: Modulation of apoptosis and cell proliferation. Toxicol Sci 52:255-261.

Wada S, Khoa TV, Kobayashi Y, Funayama T, Yamamoto K, Natsuori M and Ito N (2003) Detection of radiation-induced apoptosis using comet assay. J Vet Med Sci 65:1161-1166.

Zamora-Perez A, Camacho-Magaña B, Gómez-Meda B, Ramos-Ibarra M, Batista-González C and Zuñiga-González G (2006) Importance of spontaneous micronucleated erythrocytes in bottlenose dolphin (Tursiops truncatus) to marine toxicology studies. Acta Biol Hung 57:441-448.