Mutation Research 673 (2009) 78–81

Contents lists available at ScienceDirect

Mutation Research/Genetic Toxicology and Environmental Mutagenesis

journal homepage: www.elsevier.com/locate/gentox
Community address: www.elsevier.com/locate/mutres

Short communication

Establishment of experimental conditions for preserving samples of fish blood for analysis with both comet assay and flow cytometry

Wanessa A. Ramsdorf[a,*], Fernando de S.F. Guimarães[b], Marcos V.M. Ferraro[a], Juarez Gabardo[a], Edvaldo da Silva Trindade[b], Marta Margarete Cestari[a]

[a] Departamento de Genética, Laboratório de Mutagênese Ambiental, Universidade Federal do Paraná, Curitiba, PR, Brazil
[b] Departamento de Biologia Celular, Laboratório de Investigação de Polissacarídeos Sulfatados, Universidade Federal do Paraná, Curitiba, PR, Brazil

ARTICLE INFO

Article history:
Received 2 June 2008
Received in revised form
20 November 2008
Accepted 25 November 2008
Available online 3 December 2008

Keywords:
Comet assay
Flow cytometry
Preservation samples

ABSTRACT

When environmental analysis is performed, the high number of samples required and handling conditions during the transport of these samples to the laboratory are common problems. The comet assay is a useful, highly sensitive tool in biomonitoring. Some studies in the literature aim to preserve slides in lysis solution for use in the comet assay. Until now, however, no efficient methodology for preserving blood samples for this assay has been described. Because of this, the present report aimed to establish the proper conditions for samples maintenance prior to comet assay analysis. Samples were conserved in three different solutions: a high protein concentration solution (fetal bovine serum—FBS), an anticoagulant agent (a calcium chelator – ethylenediaminetetracetic acid – EDTA), and a salt buffered solution (phosphate buffered saline—PBS). Therefore, peripheral blood samples of Rhamdia quelen specimens were collected and maintained in these solutions until testing at 72 h. Analyses of DNA fragmentation via the comet assay and cell viability via flow cytometry were performed at intervals of 24 h. The results showed that samples maintained in FBS were preserved better; this was followed by those preserved in PBS and then last by those preserved in EDTA. In conclusion, blood samples from freshwater fish can be preserved up to 48 h in fetal bovine serum at 4 °C in the absence of light. In this period, no DNA fragmentation occurs. We thus describe an excellent method of sample conservation for subsequent analysis in the laboratory.

1. Introduction

The Advisory Committee on the Marine Environment (ACME) from the International Council for the Exploration of the Sea (ICES) recommends sampling of 25 fish/sampling sites for research studies. This situation may lead to a chaotic workload, especially because subsamples are needed for each individual [1]. Then, the fixation of biological samples represents a fundamental step. Unfortunately, such procedures often result in various degrees of artifacts [2]. To evaluate sample preservation, several assays are necessary. Comet assay is a fast and relatively inexpensive method for measuring DNA damage. This assay has thus far found many applications, particularly in biomonitoring and environmental monitoring [3]. Flow cytometry is another important analysis used to study cellular integrity [4]. Its capacity to measure large numbers of cells at a high speed makes flow cytometry useful for the screening of the micronucleus (MN) [5]. Pioneers in automating the mouse MN assay using flow cytometry described a correlation between manual microscopic and flow cytometric counts of micronucleated cells [6].

Since different methods for maintaining biological sample stability during transportation or laboratorial proceedings exist and such cell preservation is important because it allows us to perform comet assays and flow cytometry analysis in environmental toxicology studies, the aim of this study is to identify the best solution for maintaining the integrity of fish erythrocytes. Then we have used phosphate buffered saline (PBS), ethylenediaminetetracetic acid (EDTA) and fetal bovine serum (FBS). PBS is a buffer solution commonly used in biochemistry but it has many other uses as well since it is isotonic and non-toxic to cells [7]. EDTA is used for blood samples because of its function as an anticoagulant [8] and FBS is widely used in cell culture and has a high concentration of proteins.

2. Material and methods

2.1. Samples

Six specimens of silver catfish (*Rhamdia quelen*) were commercially acquired, and acclimation was performed for four weeks in aired tanks at a constant temperature (22 °C) under controlled light (12/12 dark/light). Each specimen was anaesthetized with 20% benzocaine (Merck, Darmstadt, Germany) [9], and

* Corresponding author at: Dpto. Genética, Setor de Ciências Biológicas, Jardim das Américas, UFPR, Centro Politécnico, Caixa Postal: 19071, CEP 81531-990, Curitiba, PR, Brazil. Tel.: +55 41 3361 1727; fax: +55 41 3361 1793.
E-mail address: wanessa@ufpr.br (W.A. Ramsdorf).

1383-5718/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.mrgentox.2008.11.010

peripheral blood was obtained from the dorsal vessels by vertically puncturing the axilla of the anal fin up until the hemal canal. A total of 10 μl of blood sample was stored in 1 ml of phosphate buffered saline (PBS, pH 7.4) and either 1 ml of ethylenediaminetetracetic acid (EDTA, 0.5 M) or 1 ml of fetal bovine serum (FBS). All samples were maintained at 4 °C and protected from light. After an interval of 24 h (time 0, 24, 48, and 72 h), comet assay and flow cytometry viability evaluations were performed.

2.2. Alkaline comet assay

The comet assay was performed with peripheral blood (erythrocytes) as previously described [10]. Briefly, 10 μl of the homogenized blood derived from each test solution was diluted in 120 μl of low melting agarose (LMA) and placed on a slide covered by normal agarose. The slides placed in lyses solution (lysis stock solution: NaCl (2.5 M), EDTA (100 mM), Tris (10 mM), NaOH (0.8%), N-lauryl-sarcocinate (1%); lysis working solution: triton X100 (1%), DMSO (10%) in lysis stock solution), for 24 h at 4 °C. In the following step, the slides were first immersed in a solution of NaOH (10N) and EDTA (200 mM), pH > 13 for 20 min, to effect DNA denaturation, and then subjected to electrophoresis at 300 mA and 25 V for 25 min. After neutralization in 0.4 M Tris, pH 7.5 and fixation in ethanol for 10 min, comets were stained with 0.02 g/ml ethidium bromide and scored using a Leica DMLS2 epifluorescence microscope. One hundred comets on each slide were scored visually as belonging to one of five classes, predefined with respect to tail intensity, and each scored comet was given a value of 0, 1, 2, 3, or 4 (from undamaged (0) to maximally damaged (4)) [11]. The sample dilution was necessary in detriment of the greater number of cells per microliter in fishes' blood (in this work we found 2×10^7 cells/μl).

2.3. Flow cytometry

Data acquisition and analysis were performed in focus to scatter features, debris presence, and membrane integrity in a FACScalibur flow cytometer equipped with an argon ion laser tuned to 488 nm. For all measurements, at least 20,000 cells were collected from each sample tube using CellQuest software (Becton Dickinson, San Jose, CA, USA). Data analyses were performed using CellQuest and WinMDI 2.9 software. To evaluate membrane integrity using propidium iodide (PI) uptake, 50 μl of blood sample was transferred carefully into the bottom of a test tube. PI was added to a final concentration of 50 μg/ml. Samples were gently resuspended and incubated for 10 min at room temperature in the dark. Propidium iodide was used for this reason because it is well known that stain the nucleus of cells without membrane integrity. Since PI is a supravital stain, it can distinguish between necrotic (brightly stained) and viable (negative) cells [4]. This analysis was performed on PI stained from a cell gate to exclude debris in the viability analysis.

2.4. Statistical analysis

The Kruskal–Wallis test was used in the comet assay to compare the differences between both test solutions (PBS, EDTA, and FBS) and different times (0, 24, 48, and 72 h). For flow cytometry, the obtained data were transformed into $\sqrt{x + 0.5}$ to ensure a normal distribution. These data were submitted to an analysis of variance (ANOVA) with a factorial diagram to determine statistical significance. The Tukey test was performed when the effects of interaction were significant. A p-value less than 0.05 was considered statistically significant.

3. Results and discussion

Some reports have suggested the possibility of storing slides in lysis buffer for periods up to four months [12]. However, reports developed by Belpaeme et al. (1998) have shown a possible effect of storing slides in lysis solution, that produce changes in the comet patterns in samples stored for a prolonged period [1].

For the analysis of the optimal solution for sample conservation, the comet assay was performed to evaluate possible DNA damage in blood cells up to 72 h after collection, and compare the samples in different test solutions. Previous observations show that fish blood

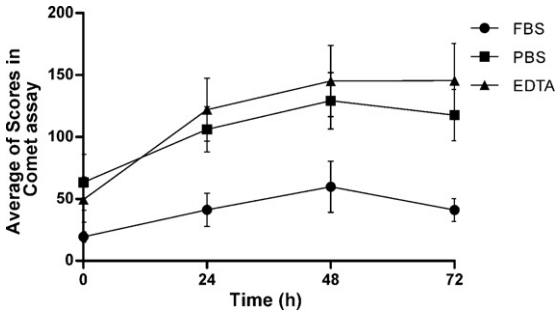

Fig. 1. Comparison of the comet assay responses (scores) of blood cells for different incubation times (0, 24, 48, and 72 h) and solutions (FBS, PBS, and EDTA).

samples collected with heparin and escorted to laboratory in ice, coagulates. The statistic analysis of comet assay detected significant differences between the DNA damage induced by conservation in FBS and PBS, and differences in DNA preservation were noted between FBS and EDTA as well (0 h: $p = 0.0201$; 24 h: $p = 0.0285$; 48 h: $p = 0.0484$; 72 h: $p = 0.015$ to FBS and PBS and 0 h: $p = 0.0399$; 24 h: $p = 0.0119$; 48 h: $p = 0.0248$; 72 h: $p = 0.0035$ to FBS and EDTA). However, difference between PBS and EDTA was not observed (0 h: $p = 0.7869$; 24 h: $p = 0.7456$; 48 h: $p = 0.7869$; 72 h: $p = 0.6265$). At all times tested, cells preserved in FBS always presented the lowest genetic material damage in comparison to the other solutions (Fig. 1).

The comet assay also allowed us to verify which periods and solutions provided the best blood storage modality for our cell suspensions. The blood samples conditioned either in FBS ($p = 0.1509$) or in PBS ($p = 0.1597$) did not exhibit differences in damage between the testing times. While samples conditioned in EDTA did not present damage differences between time 0 and 24 h ($p = 0.0724$), 24 h and 48 h ($p = 0.4379$), 24 h and 72 h ($p = 0.4877$), and 48 h and 72 h ($p = 0.9349$), they did show significant differences between time 0 and 48 h ($p = 0.0101$), 0 and 72 h ($p = 0.0128$), with timepoints at 48 and 72 h displaying the highest DNA damage (Fig. 1).

Flow cytometry analysis was performed at the timepoints (0, 24, 48, and 72 h) in order to identify the timing of changes in scatter features (P1) and viability (P2). P1 analysis was performed with the aim of detecting apoptotic cells [4]. The ANOVA clearly showed that there were differences between the storage solutions ($p < 0.01$). The Tukey test revealed differences between PBS and EDTA as well as FBS and EDTA. Then the FBS presented the highest averages, indicating minor morphologic scatter changes in cells. This was followed by PBS and then EDTA (Table 1). P1 analysis still revealed an effect of sampling time, with 72 h being different than the other groups. An interaction for time and solution was not detected. These differences observed only at 72 h indicated by the lower media levels of morphologically intact cells.

The P2 analysis was performed by using propidium iodide in order to verify cellular viability. The ANOVA showed a difference between the treatments ($p < 0.01$), indicating a solution effect as well as a time effect. However, we did not observe an interaction

Table 1
Flow cytometry: differences between solutions and differences between times.

	Solutions			Times			
	PBS	EDTA	FBS	0 h	24 h	48 h	72 h
Average transformed of P1	122.43^{a}	46.51^{b}	127.14^{a}	105.23^{A}	102.16^{A}	99.78^{A}	87.61^{B}
Average transformed of P2	40.05^{c}	43.16^{c}	24.74^{d}	27.72^{C}	34.73^{CD}	39.14^{CD}	42.34^{D}

Cytometry data were transformed with $\sqrt{x + 0.5}$, where x was the absolute value of the number of events in 20,000 cells without changes in scatter features (P1) and without viability (P2). Different solutions (PBS, EDTA and FBS) were analyzed over different times (0 h, 24 h, 48 h and 72 h). Small superscript letters (a–d) indicate comparison between solutions and capital superscript letters (A–D) indicate comparison between times, in all situations $p < 0.01$. The same letters indicate the lack of a statistically significant difference, and different letters indicate a statistically significant difference.

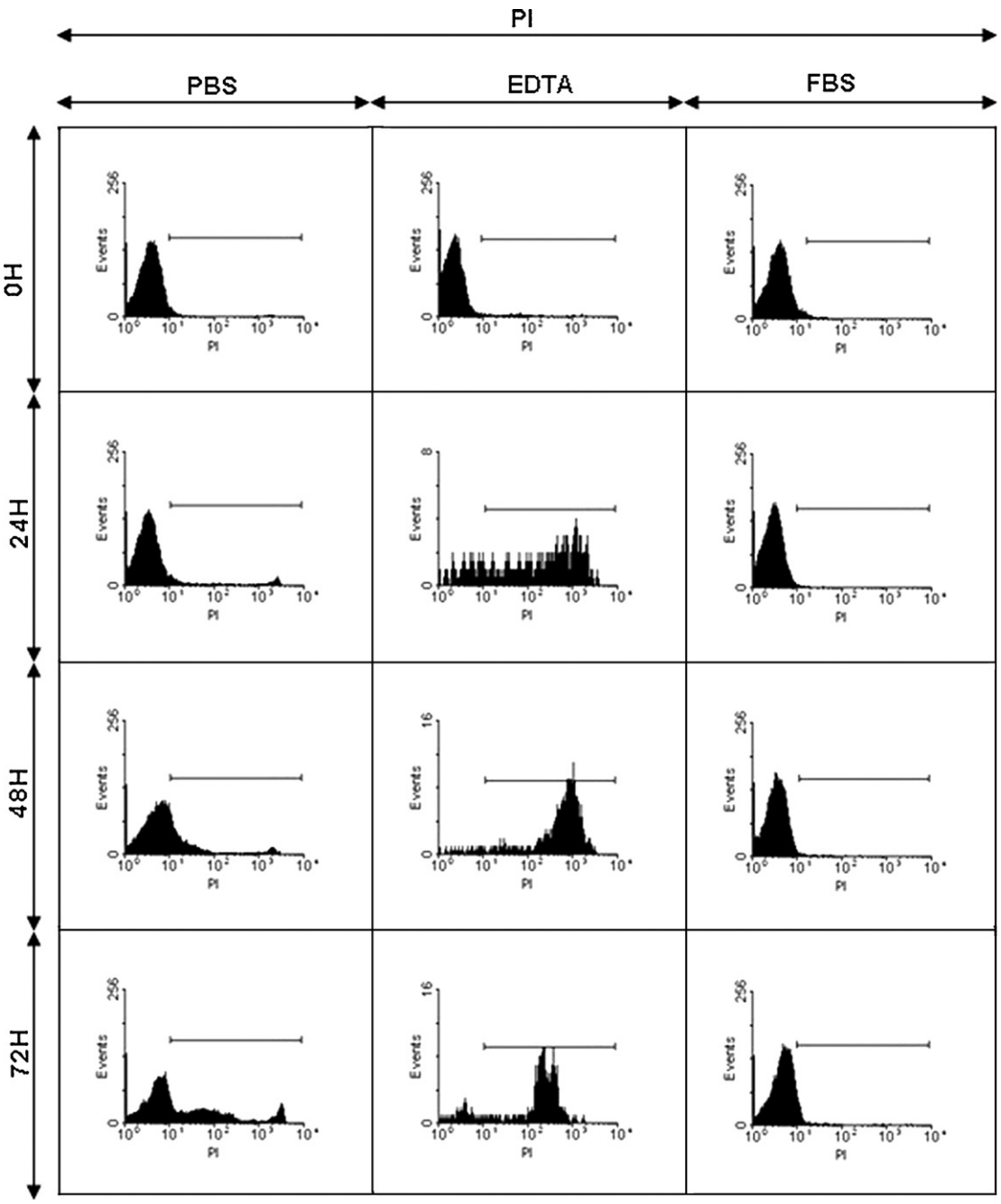

Fig. 2. PI viability of cells gated from R1. Histograms indicate unviable cells dislocated under the bars.

between time and solution. There was no difference between the PBS and EDTA solutions, but differences were present for EDTA and FBS as well as PBS and FBS. EDTA was the solution with the higher number of unviable cells. PBS had fewer unviable cells, and FBS had the lowest number of unviable cells (Table 1). The Tukey test showed a difference between the timepoints of 0 and 72 h; the intermediate periods (24–48 h) were similar to both the initial and final timepoints. Fig. 2 shows unviable cells dislocated under the bars.

When the different solutions were compared, we observed that FBS best preserved the fish blood samples collected. There were no significant differences between preservation samples until 72 h after collection; at this point, cells preserved in FBS showed lower DNA damage than cells preserved in the other two solutions. Our results suggest that PBS and EDTA are not sufficient for preserving cell genetic material over time.

These data together show that FBS best preserved both cells and their genetic material. Preservation in PBS was statistically similar to that in FBS in the P1 (morphology analysis), suggesting that PBS provoked little morphological alteration. However, the P2 analysis showed that the cells were unviable. These data confirm the results obtained by comet assay, which showed significantly more DNA breaks in cells maintained in PBS than in FBS. These results may be related to the low osmolarity of PBS (149.14 mM) in comparison to that usually found in cells of freshwater fish (i.e., 292.5 mM in *Salmo trutta* [13]). The osmolarity difference between blood and PBS could have led to a water influx in cells that did not undergo either alteration in their morphology or debris formation. The nucleus may have thus been affected (expelled), reducing cellular viability.

EDTA was used for blood sample preservation because of its function as an anticoagulant. This agent sequesters calcium ions,

important cofactors of several serine proteases of the blood coagulation cascade, and it is, therefore, a good anticoagulant [8]. However, our data showed that EDTA is not a good solution for preserving the samples analyzed. In cytometry assays, we observed that EDTA slowly leads to damage in genetic material of the cells in suspension (Table 1). This finding supports studies that verified the progressive and irreversible loss of antigen-specific lymphoproliferative responses in peripheral blood mononuclear cells obtained from blood exposed to EDTA [8]. Moreover, a report made by Machella et al., 2006 showed that when DNA of teleost fish (seabass *Dicentrarchus labrax*) was extracted and maintained in the presence of EDTA, this compound also could not reduce basal levels of DNA strand breaks [14].

FBS was the optimal solution tested. This characteristic may be due to its composition. The serum is a rich source of proteins, growth factors, amino acids, carbohydrates, ions, vitamins and other compounds. Several components of the serum are likely beneficial for protecting the blood samples in this study. Moreover, the high quantity of glucose present in the serum (~0.6–1.2 mg/ml) [15] could serve as an energy source for cellular metabolism [16]. The presence of protease inhibitors is particularly important because of their anti-trypsin activity, which inhibits this enzyme to act in blood cells. Additionally, the osmolarity of the serum (322 mM [15]) was similar to that of the blood of freshwater fish (292.5 mM in *Salmo trutta* [13]). Therefore, this solution is suitable for this cell type. However, further evaluation may reveal that this solution is not the best option for marine organisms (osmolarity 1118.32 mM in *Myxine* blood, hagfish [13]).

Interestingly, the samples maintained in FBS for 72 h showed minor DNA damage that was not present at 48 h. Similar data were obtained for PBS. Since the comet assay is a very sensitive method, these data suggest that DNA repair may occur in the cells during the time evaluated [17].

We thus conclude that fetal bovine serum best preserved the blood cells and genetic material of freshwater fish for times ranging from 0 to 48 h. Then we recommend its use (at 4 °C in the absence of light) for maintaining blood collected from fish in the field when immediate processing of the material for comet assay biomonitoring is impossible.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgements

The authors thank CNPq, CAPES, SETI-PR, Fundação Araucária, and the Aquatóxi group.

References

[1] K. Belpaeme, K. Cooreman, M. Kirsch-Volders, Development and validation of the in vivo alkaline comet assay for detecting genomic damage in marine flatfish, Mutat. Res. 415 (3) (1998) 167–184.
[2] J. Dubochet, N. Sartori Blanc, The cell in absence of aggregation artifacts, Micron 32 (1) (2001) 91–99.
[3] G. Frenzilli, M. Nigro, B.P. Lyons, The comet assay for the evaluation of genotoxic impact in aquatic environments, Mutat. Res.: Rev. Mutat. Res. (2008), doi:10.1016/j.mrrev.2008.03.001.
[4] B. Canonico, L. Zamai, S. Burattini, V. Granger, F. Mannello, P. Gobbi, C. Felici, E. Falcieri, J.T. Reilly, D. Barnett, S. Papa, Evaluation of leukocyte stabilisation in TransFix®-treated blood samples by flow cytometry and transmission electron microscopy, J. Immunol. Methods 295 (2004) 67–78.
[5] P. Sánchez, M.T. Llorente, A. Castaño, Flow cytometric detection of micronuclei and cell cycle alterations in fish-derived cells after exposure to three model genotoxic agents: mitomycin C, vincristine sulfate and benzo(a)pyrene, Mutat. Res. 465 (2000) 113–122.
[6] K.J. Hutter, M. Stohr, Rapid detection of mutagen induced micronucleated erythrocytes by flow cytometry, Histochemistry 75 (3) (1982) 353–362.
[7] J. Stenesh, Biochemistry, Plenum Press, New York, 1998.
[8] P. Kumar, V. Satchidanandam, Ethyleneglycol-bis-(β-aminoethylether) tetraacetate as a blood anticoagulant: preservation of antigen-presenting cell function and antigen-specific proliferative response of peripheral blood mononuclear cells from stored blood, Clin. Diagn. Lab. Immunol. 7 (4) (2000) 578–583.
[9] L.C. Gomes, A.R. Chippari-Gomes, N.P. Lopes, R. Roubach, C.A.R.M. Araújo-Lima, Efficacy of benzocaine as an anesthetic in juvenile tambaqui *Colossoma macropomum*, J. World Aquacult. Soc. 32 (4) (2001) 426–431.
[10] M.V. Ferraro, A.S. Fenocchio, M.S. Mantovani, M.M. Cestari, C.A.O. Ribeiro, Mutagenic effects of tributyltin (TBT) and inorganic lead (PbII) on the fish *H. malabaricus* as evaluated using the comet assay, piscine micronucleus and chromosome aberrations tests, Genet. Mol. Biol. 27 (2004) 103–107.
[11] A. Collins, M. Dušinská, M. Franklin, M. Somorovská, H. Petrovská, S. Duthie, L. Fillion, M. Panayiotidis, K. Rašlová, N. Vaughan, Comet assay in human biomonitoring studies: reliability, validation, and applications, Environ. Mol. Mutagen. 30 (2) (1997) 139–146.
[12] D.E. Nacci, S. Cayula, E. Jackim, Detection of DNA damage in individual cells from marine organisms using the single cell gel assay, Aquat. Toxicol. 35 (1996) 197–210.
[13] W.T.W. Potts, G. Parry, Osmotic and Ionic Regulation in Animals, Pergamon, Oxford, 1964.
[14] N. Machella, M. Battino, B. Pisanelli, F. Regoli, Influence of the SCGE protocol on the amount of basal DNA damage detected in the Mediterranean mussel, *Mytilus galloprovincialis*, Environ. Mol. Mutagen. 47 (2006) 579–586.
[15] H.R. Maurer, Towards chemically-defined, serum-free media for mammalian cell culture, in: R.I. Freshney (Ed.), Animal Cell Culture: A Practical Approach, Oxford, IRL Press, 1986, pp. 13–30.
[16] K.L. Scott, J. Lecak, J.P. Acker, Biopreservation of red blood cells: past, present, and future, Transf. Med. Rev. 19 (2) (2005) 127–142.
[17] E.E. Visvardis, A.M. Tassiou, S.M. Piperakis, Study of DNA damage induction and repair capacity of fresh and cryopreserved lymphocytes exposed to H_2O_2 and γ-irradiation with the alkaline comet assay, Mutat. Res. 383 (1997) 71–80.