Applications of Different Comet Assay (the Single Cell Gel Eelectrophoresis) Methods for Detecting DNA Damage in Cryopreserved Fish Sperm

Kriyoprezervasyon Uygulanmış Balık Sperm Hücrelerinde DNA Hasarının Tespitinde Farklı Comet Analizi (Tek Hücre Jel Elektroforezi) Metotlarının Uygulanmaları

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Abstract

Cryopreservation as an assisted reproduction method is commonly used for successful long-term storage of not only human but also animal gametes, even their embryos. Moreover, cryopreservation of sperm offers many advantages. In fish, for instance, it is useful for improving artificial reproduction, facilitating genetic manipulation, and reducing the amount of male breeders stocks. However, after cryopreservation, sperm cell could have some problems such as loss in motility and DNA damage, leading to lower fertilization and hatching rates. The comet assay is a simple, but sensitive tool for detecting strand breaks in DNA in single sperm cells. In this study, three comet assay methods which contain different chemical agents and have different application times were carried out on fish sperm. The mixed sperm samples obtained from mature carp (*Cyprinus carpio*) males were used in the experiments. Firstly, using fresh sperm samples, DNA damage is classified by means of lysis solutions of each used method without H_2O_2 and with 12, 25 and 50 µM H_2O_2. Thus, DNA damage is visually scored by measuring the DNA tail of each sperm cell in fluorescent microscope images as positive control (undamaged DNA), 1, 2, and 3 classes of damage, referring from the less to more damaged sperm cells. Secondly, after thawing, cryopreserved sperm samples were applied by the comet assay methods. The results of three methods have shown no significant differences among the percentages of undamaged DNA (67.7±2.0 %, 69. 3±1.8 % and 68.2±2.8 %) and total damaged DNA (32.3±2.0 %, 30.7±1.8 % and 31.8±2.8 %) of sperm cells as. On the contrary, there were some statistical differences among the classes of damage. Consequently, all methods have been found successful in detection of undamaged and total damaged DNA but not in the recognition of the damage classes.

Key Words: Cryopreservation, Comet Assay, DNA damage, Fish sperm, Single Cell Gel Eelectrophoresis

Özet

Kriyoprezervasyon, hem insan hem de hayvan gametlerinin, hatta embriyolarının, başarılı bir şekilde uzun süreli saklanması için yaygın olarak kullanılan yardımcı üreme tekniklerindendir. Ayrıca, sperm kriyoprezervasyonun birçok avantajı vardır. Örneğin, balıklarda suni döllemenin geliştirilmesinde, genetik manipülasyonların kolaylaştırılmasında, erkek anaç stokunun azaltılmasında kullanılır. Ancak kriyoprezervasyon sonrası, sperm hücrelerinde sonrasında dölleme ve yumurtadan çıkış oranlarının azalmasına sebep olacak motilite kaybı ve DNA hasarı gibi problemler ile oluşabilir. Comet analizi, DNA sarmalındaki kırıkları tek bir sperm hücresi seviyesinde tespit edebilen basit ve hassas bir araçtır.

Bu çalışmada, farklı kimyasal maddeler ve uygulama zamanlarına sahip üç farklı Comet analiz metodu balık sperm hücrelerine uygulanmıştır. Olgun sazan balığı (Cyprinus carpio) bireylerinden alınan sperm örnekleri bu çalışmada kullanılmıştır. Öncelikle, taze sperm örnekleri kullanılarak, her metot için liziz solüsyonuna 12, 25 ve 50 µM H_2O_2 eklenerek DNA hasarı sınıflandırılmıştır. Böylece, DNA hasarı, her hücre DNA'sının oluşturduğu kuyruk uzunluğuna göre floresan mikroskop altında incelenerek görsel olarak, 1. 2. ve 3. dereceden hasarlı ve H_2O_2 eklenmemiş örnekler 0 (hasarsız) olarak ölçeklendirilebilir. Sonrasında, kriyoprezervasyon uygulanmış sperm örnekleri çözündürülerek, üç farklı Comet analiz metodu uygulanmıştır. Üç metoda ait yüzde hasarsız DNA sonuçları (%67,7±2,0; % 69,3±1,8 ve %68,2±2,8) arasında ve toplam hasarlı DNA sonuçları (%32,3±2,0; % 30,7±1,8 ve %31,8±2,8) arasında istatistiki olarak önemli derece fark saptanamamıştır. Buna karşın, DNA hasar sınıflandırmaları arasında bazı istatistiksel farklılıklar bulunmaktadır. Sonuç olarak, her üç metot hasarsız ve toplam hasarlı DNA tespitinde benzer başarıyı göstermekte iken DNA hasar sınıflarının tanımlanmasında farklılıklar olabilmektedir.

Anahtar kelimeler: Kriyoprezervasyon, COmet Analizi, DNA Hasarı, Balık Spermi, Tek Hücre Jel Elektroforezi

Introduction

In reproductive biology, cryopreservation is a common method to preserve sperm cells, and has been applied in sperm of different animal groups as well as in fish sperm (Barbas & Mascarenhas, 2009; Diwan et al., 2010). The cryopreservation of sperm offers many benefits. In fish, for instance, the cryopreservation could be used in conservation of endangered fish species, acquiring desired genotype through cross-breeding, easy transportation of genetic material, broodstock protection, optimal sperm utilization in hatchery production and laboratory experiments, easy transportation of genetic material among hatcheries, economically and effectively maintenance of breeders in hatcheries, experimental material for advanced studies (Chao *et al*., 1987; Chao& Liao, 2001).

Conceptually, the procedure of cryopreservation could be divided into two main processes; the freezing and the thawing. These processes have potential to damage sperm cells associated with loss of motility and accordingly fertility rate, loss of plasma membrane, the integrity mitochondria and DNA (Cabrita et al., 1998; Suquet et al., 1998). Damages in DNA as a result of the cryopreservation procedure were determined not just in human sperm also in boar, ram, and equine sperm by comet assay (Hughes et al., 1997; Baumber et al., 2003; Soliman et al., 2004; Fraser & Strzeżek, 2005). Similar damages in DNA have been found in different fish species such as rainbow trout (Oncorhynchus mykiss), gilthead sea bream (Sparus aurata), loach (Misgurnus fosilis), sea bass (Dicentrarchus labrax) (Zilli et al., 2003; Kopeika et al., 2003; Cabrita et al., 2005;).

The comet assay, or also known as single-cell gel electrophoresis, is one of the common methods for assessing DNA damage at individual cell level (Ostling & Johanson, 1984). There are several different types of comet assays which mostly vary based on the pH lysis and electrophoresis solutions (Nossoni, 2008). The procedure of comet assay demonstrated in Fig. 1 has seven main steps; isolation of cells, preparation of slides, lysis, unwinding of DNA strands, electrophoresis, neutralization, fluorescent staining and microscopic evaluation.

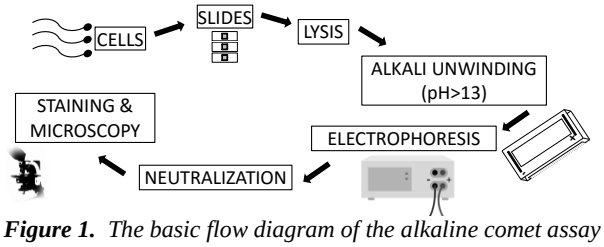

Figure 1. *The basic flow diagram of the alkaline comet assay procedure, indicating main steps associated with the assay (modified from Tice et al. 2008).*
Şekil 1*. Comet analizi temel prosedürünün temel basamaklarını belirten akış şeması (Tice vd. (2008)'den değiştirilerek).*

In this study, three comet assay methods, Singh et al. (1988), Cabrita et al. (2005), Shen & Ong, (2000), which contain different chemical agents and have different application times were carried out on fish sperm. These three methods have been compared with regards to their detection capacity in DNA damage scores.

Materials and Methods

In this study, the mixed sperm samples that obtained from mature carp (*Cyprinus carpio*) males manual abdominal stripping while avoiding any contamination from water, blood, urine, or faeces were used in the experiments. The experiments were carried out in two steps. Firstly, using fresh sperm samples, DNA damage is classified by means of lysis solutions of each used method without H_2O_2 and with 12, 25 and 50 µM H_2O_2. Thus, DNA damage is visually scored by measuring the DNA tail of each sperm cell in fluorescent microscope images as positive control (undamaged DNA), 1, 2, and 3 classes of damage, referring from the less to more damaged sperm cells. Secondly, after thawing, the comet assay methods were applied by the cryopreserved sperm samples.

Fresh and frozen/thawed sperm were rinsed with phosphate buffer saline (PBS) solution, and diluted until a final concentration of 8–10 $x10^6$ sperm per ml. Slides were prepared one day before the experiments applying a thin layer of normal melting point agarose, eliminating agarose in excess. The slides were stored at room temperature and protected from dust and light. Low melting point agarose was prepared next day for the second layer. This agarose was mixed with 30 µl sperm suspension in an Eppendorf tube. The agarose: sperm suspension was added to the slides covering with a coverslip. After that, the coverslip was removed and slides were left to solidify at 4 °C for 10min. For each sample, three slides were prepared and examined. Then, three comet assay methods, Singh et al. (1988), Cabrita et al. (2005), Shen & Ong, (2000), are applied to the sample, which main differences are summarized in Table 1. In all methods, for comet visualization 40 µl ethidium bromide at final concentration 0.5 µg/ml were pipetted into the sample, and the slides were examined with a system combining a fluorescence Zeiss Axio Scope A1 (Germany) microscope with AxioCam ICc 5 camera. 100 cells were scored per slide in triplicate.

In cryopreservation procedure, the semen samples were mixed in a ratio of 1:9 (v/v) with an extender composed of modified Kurokura solution (62 mM NaCl, 134 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, and 2 mM $NaHCO_3$, pH 8.2),10% DMSO, and 10% egg yolk (Magyary et al., 1996). After dilution, the samples were drawn into 0.25 ml straws (IMV, France) and sealed with polyvinyl alcohol. The straws were placed on a rack 2.5 cm above a liquid nitrogen surface for 10 min and then plunged directly into the liquid nitrogen. At least seven straws per a sperm sample were frozen. After a week of storage in liquid nitrogen, the samples were thawed in a 20°C water bath for 30 s (Öğretmen & İnanan, 2014). The results are shown as the means ± the standard deviation (SD). Non-parametric Mann–Whitney U tests followed by Kruskall–Wallis test were used, and $\alpha<0.05$ were taken to indicate a significant difference between the treatments in terms of percentages of DNA damages.

Results and Discussion

The classification of DNA damaged cells were representative in Fig. 1. To obtain this classification, fresh samples were treated by the different concentrations of H_2O_2 in lysis solution. Under the fluorescence microscope, the sperm samples treated with lysis solution with H_2O_2 free were observed spherical in diameter between 24-30 µm. DNA of

sperm samples exposed by lysis solution with the different concentrations of H_2O_2 were tailed, and length of the tail DNA increased with the higher concentrations of H_2O_2. Thus, the sperm samples exposed by 12 µM H_2O_2 were visualized as an olive tail in 30-40 µm, and scored as grade 1 mild damaged DNA. Similarly, the sperm treatments with 25 and 50 µM H_2O_2 have image formations in the fluorescence microscope grade 2 moderate damaged DNA (length between 40-50 µm) and grade 3 severe damaged DNA (length between >50 µm), respectively.

The three different comet assay methods were applied with the different concentrations of H_2O_2 in lysis solution to reveal that there is any difference in their scaling or classification of DNA damaged (Fig. 3). There were no significant differences between the percentage of both DNA undamaged and damaged cell among the three methods ($P>0.05$). Each H_2O_2 treatments were reflected a grade of DNA damaged.

Table 1. *The main differences in the steps of among the comet assay methods used in this study (Si;Singh et al., 1998: Ca;Cabrita et al., 2005: Sh;Shen & Ong, 2000).*

Tablo1. *Bu çalışmada kullanılan COmet analizi metotlarının basamakları arasındaki temel farklılıklar (Si;Singh vd., 1998: Ca;Cabrita vd., 2005: Sh;Shen ve Ong, 2000).*

The steps	Si	Ca	Sh
Gel setting on slides	*Bottom layer;* 0.5% NMA[1] *Top layer;* Sample mixed with 0.5% LMA[2]	*Bottom layer;* 0.5% NMA *Top layer;* Sample mixed with 0.5% LMA	*Bottom layer;* 0.75% NMA *Middle layer;* Sample mixed with 0.75% LMA *Top layer;* 0.75% LMA
Lysis solution	2.5 M NaCl, 100 mM Na_2-EDTA, 10 mM Tris, 1% N-Lauroylsarcosine 1% Triton X-100. pH 10, for 1 h at 4 °C	2.5 M NaCl, 100 mM Na_2-EDTA, 10mM Tris, 1% N-Lauroylsarcosine 1% Triton X-100. pH 10, for 1 h at 4 °C	2.5 M NaCl, 100 mM Na_2-EDTA, 10 mM Tris, 1% N-Lauroylsarcosine, 1% Triton X-100. pH 10, for 1 h at 4 °C
DNA decondensation		+ 10 mM dithiothreitol for 30 min at 4 °C. + 4 mM lithium diiodosalicylate for 90min	+ RNase treatment[3] for 4 h + proteinase K[4] treatment for 15 h
Electrophoresis solution	1 mM N_2-EDTA, 300 mM NaOH	1 mM Na_2–EDTA, 300 mM NaOH	100 mM Tris, 300 mM sodium acetate
Unwinding of DNA	pH >13 for 20 min at 4 °C	pH 12 for 20 min at 4 °C	pH 10 for 20 min at 4 °C
Electrophoresis running	for 20 min at 25 V,4 °C.	for 10 min at 25 V, 300 mA, 4 °C.	for 1 h at 12 V, 100 mA, 4°C
Neutralization	0.4 M Tris, pH 7.5 for 5 min at 4 °C.	0.4M Tris, pH 7.5 for 5min at 4 °C.	0.4 M Tris-HCl pH 7.4 for at least 5 min.
Drying of slides		methanol for 3min.	

[1]NMA: normal melting agarose; [2]LMA; low melting temperature agarose; [3]RNase solution: 2.5 M NaCl, 5 mM Tris, 0.05% N-Lauroylsarcosine, pH 7.4, with 10 mg/ml RNase A; [4]proteinase K solution: 2.5 M NaCl, 5 mM Tris, 0.05% N-Lauroylsarcosine, pH 7.4, with 200 mg/ml proteinase K.

The percentages of undamaged DNA were found 98.8±0.8 %, 98.8±0.6 %, and 98.8±0.8 % with three comet assay methods, Singh et al. (1988), Cabrita et al. (2005), Shen & Ong, (2000), respectively. In the same

samples, also the percentages of Grade 1 damaged DNA were detected by all methods as < 2%. In the samples treated with 12 µM H_2O_2, According to the methods, the percentages of Grade 1 damaged DNA were scored as 78.7±0.8 %, 21.3±0.8 %, and 78.7±1.8 %. In the same samples, the percentages of Grade 2 damaged DNA were determined as 21.3±1.8 %, 78.8±1.8 %, and 21.2±1.8 %. In the samples treated with 25 µM H_2O_2, mainly the percentages of Grade 2 damaged DNA was determined as 83.8±1.5 %, 85.3±3.3 %, and 85.0±2.3%. In the same samples, the percentages of Grade 1 and Grade 3 damaged DNA were also found as < 10 %. In the samples treated with 50 µM H_2O_2, the percentages of Grade 3 damaged DNA were scored as 92.0±1.3 %, 93.5±1.3% and 92.8±1.0% with three methods (Fig. 3).

The results of the applications of three methods to cryopreserved sperm were shown in Table 2. According to the methods, the percentages of undamaged DNA were calculated as 67.67±2.0 %, 69.33±1.8 % and 68.17±2.8 % in the cryopreserved sperm. On the other hand, the percentage of total damaged DNA was found as around 30 % with all methods. The only significant differences were observed in the grading of total damaged DNA ($P<0.05$)

Eventually, the remarkable difference among the methods was appeared the pH of electrophoresis solutions. The electrophoresis solution of the method used in Singh *et al.* (1988) was >13, while the others were less tan this value. According to experience gained in the current study, the final cell number and the thickness of agarose gel added to the slides are very critical points in comet assay application of sperm cells. These two factors are directly responsible for the clear visualizing of DNA fragments to be scored in the fluorescent microscope.

It is obvious that DNA damage as a cryoinjury can be detected by different comet assay procedures. Also, both sperm samples treated by H_2O_2 and cryopreserved have shown different resistance behaviors at cell level in terms of DNA damages. The relationships between DNA damages and fertility, and also hatching ratios and malfunctions in offspring should be investigated in the further studies. Recently, with the popularity of gene and sperm banking of animal species, cryopreservation and the detection of cryoinjuries like DNA have become more important. Especially, for this kind of banking, it should be clearly viewed that effects of the different DNA damage grades on next progeny.

Conclusions

Even though the advantages of usage of cryopreserved sperm in artificial insemination, detection of DNA damages in sperm could turn out to be a critical factor. In this study, three comet assay applications suggested by previously studies have been compared. All methods have been found successful in detection of undamaged and total damaged DNA but not in the recognition of the damage classes. Consequently, the comet assay method suggested by Singh *et al.* (1988) is useful than the methods suggested by Cabrita *et al.* (2005) and Shen & Ong, (2000) in terms of consuming less chemical agent and having a shorter application time.

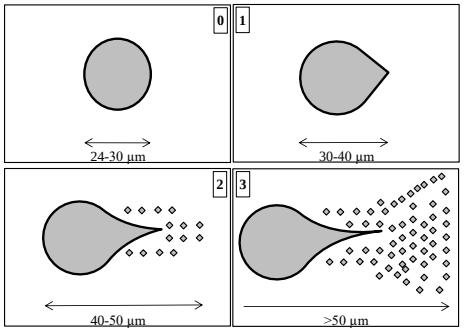

Figure 2. *The figuration of comet images showing different levels of damage according to different-sized tails in visual scoring; score 0 (undamaged DNA);grade 1 (mild damaged DNA); grade 2 (moderate damaged DNA); grade 3 (severe damaged DNA).*
Şekil 2. *Farklı kuyruk uzunluklarına göre DNA hasar seviyelerinin görsel Comet görüntülerinin şematize edilmesi: 0; hasarlanmamış DNA, 1; az hasarlı DNA, 2; hasarlı DNA, 3; çok hasarlı DNA.*

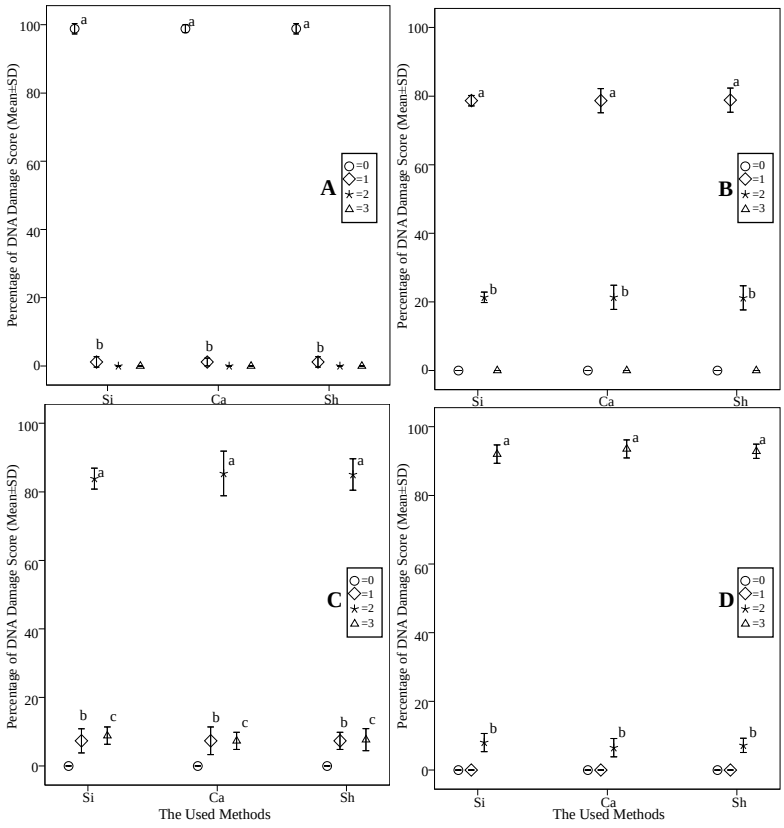

Figure 3*. The classification of DNA damage scores (0; undamaged and various degrees of damage from minor to severe, 1, 2, and 3). To obtain this scale, the different concentrations of H_2O_2 added to lysis solution (A; lysis solution with H_2O_2 free, B; lysis solution with 12 µM H_2O_2, C; lysis solution with 25 µM H_2O_2, D; lysis solution with 50 µM H_2O_2) using different methods (Si;Singh et al., 1998: Ca;Cabrita et al., 2005: Sh;Shen & Ong, 2000). The letters show significancy at α= 0.05.*
Şekil 3. *DNA hasar derecelerinin sınıflandırılması (0; hasarlanmamış DNA, 1; az hasarlı DNA, 2; hasarlı DNA, 3; çok hasarlı DNA). Bu ölçeklendirme farklı metotların (hasarları (Si; Singh vd., 1998: Ca;Cabrita vd., 2005: Sh;Shen ve Ong, 2000)) liziz solüsyonlarına H_2O_2 eklenmeden (A), 12 µM H_2O_2 (B), 25 µM H_2O_2 (C) ve 50 µM H_2O_2 (D) eklenerek elde edilmiştir. Aynı harfler aralarında fark bulunmayan (P>0,05) benzer grupları göstermektedir.*

Table 2. *The percentages of DNA damages of cryopreserved fish sperm obtained from different methods (Si;Singh et al., 1998: Ca;Cabrita et al., 2005: Sh;Shen & Ong, 2000). Grade 1; mild undamaged DNA, Grade 2; moderate undamaged DNA, Grade 3; severe undamaged DNA. The same letter shows no significance at the 5% level.*

Tablo 2. *Farklı metotlar kullanılarak elde edilen kriyoprezervasyon uygulanmış balık sperm hücrelerinde yüzde DNA hasarları (Si; Singh vd., 1998: Ca;Cabrita vd., 2005: Sh;Shen ve Ong, 2000). 0; hasarlanmamış DNA, 1; az hasarlı DNA, 2; hasarlı DNA, 3; çok hasarlı DNA. Sütunlardaki aynı harfler aralarında fark bulunmayan (P>0,05) benzer grupları göstermektedir.*

		Total damaged			
Methods	**undamaged DNA**	**DNA**	**Grade 1**	**Grade 2**	**Grade 3**
Si	67.67±2.0[a]	32.3±2.0[a]	15.0±2.0[a]	13.0±1.8[a]	4.3±2.5[a]
Ca	69.33±1.8[a]	30.7±1.8[a]	7.8±1.8[b]	11.8±2.4[a]	11.0±1.0[b]
Sh	68.17±2.8[a]	31.8±2.8[a]	4.3±1.3[b]	12.2±1.3[a]	15.3±0.3[c]

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