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Original Article

Outcomes of comet assay analysis using freshly prepared and cryopreserved leukocytes

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Abstract

Comet assay analysis is a useful method which can be used to quantify systemic oxidative stress. Different laboratories use slightly different approaches when performing comet assay analysis. While some use cryopreserved leukocytes, others perform comet assays on freshly prepared cells. The purpose of this investigation was to compare comet assay results for single-stranded (ss)-DNA breaks in freshly prepared and cryopreserved leukocytes. A comparative quantification of ss-DNA breaks was performed on circulating leukocytes from two groups of ten healthy subjects (age- and sex-matched). Comet assay analysis of ss-DNA breaks was performed on all individuals on the same day. In the first group of ten subjects (group 1), leukocytes were isolated from the whole blood and comet assay was directly performed on the freshly isolated leukocytes. In the second group of ten subjects (group 2), blood samples were taken the day before comet assay analysis and the leukocytes were isolated and cryopreserved for 24 h. DNA damage (ss-DNA breaks) was quantified in the two groups, and the values of DNA breaks were calculated as comet tail moment. As result, group 2, where cryopreserved cells were used for comet assay analysis, had a significantly higher amount of ss-DNA damage in the circulating leukocytes than group1, in which freshly prepared leukocytes were used for analysis (tail moment: 3.28 AU in comparison to 0.38 AU, respectively). In conclusion, cryopreservation of leukocytes increases the number of ss-DNA breaks, and therefore, affects the results of comet assay analysis. Performing comet assay analysis on freshly prepared leukocytes is an approach that is likely to yield more accurate results.

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INTRODUCTION

Twenty-five years ago, two Swedish scientists, namely stling and Johanson [1], developed a new method for analysing DNA damage. This method, used in biological research, imports the word ‘comet’ from the realm of astronomy as its name. This method quantifies DNA breaks. The concept behind ‘comet assay’ methodology is that undamaged DNA retains a highly organized association with matrix proteins in the nucleus. When damaged, this organization is disrupted. When an electric field is applied to the DNA, which has an overall negative charge, it is drawn towards the anode, which is positively charged [2]. Intact DNA (*i.e.* no DNA breaks) is such a large molecule that it hardly moves when exposed to an electrical field. DNA breaks, however, lead to smaller pieces of DNA which migrate away from the intact DNA nucleus. The amount of migrated DNA is a measure of the extent of DNA damage [3]. This method is currently being increasingly used to indirectly quantify oxidative stress, as oxidative stress is a well-recognized pathogenetic mechanism for various diseases [4-7].

Oxidative stress in cells is caused by an imbalance between the production of reactive oxygen species (ROS) and our biological system's ability to neutralize these ROS and repair the resulting damage [8]. We are constantly exposed to factors which induce the production of ROS (*e.g.* UV irradiation, blood flow disturbances, chemicals) [9]. Under optimal conditions, the magnitude of ROS formation is balanced by the rate of ROS elimination by the diverse antioxidants in our bodies [10-12]. If, however, the production of ROS is larger than a body’s coping capacity, the resulting oxidative stress damages cell structures and macromolecules such as DNA [13]. Fortunately, nature has also developed mechanisms to repair (*e.g.* DNA) or

eliminate (*e.g*. proteins via proteasomes) damaged molecules. If this capacity is also exhausted, the damage accumulates over time, leading to clinically relevant damage known as 'disease' [14].

In humans, oxidative stress is involved in many diseases [9, 15-19]. Oxidative stress plays an important role in the pathogenesis of neurodegenerative diseases, including Parkinson's disease [20], Alzheimer's disease [21], and Huntington's disease [22]. Oxidative stress is thought to play a role in certain cardiovascular diseases, since oxidation of LDL in the vascular endothelium is a precursor to plaque formation [23, 24]. Oxidative stress has become increasingly accepted as playing a role in the aging process [25] as well as in the development of cancer [26, 27]. It is also known to be a well-recognized pathogenetic mechanism leading to various ophthalmic diseases, such as age-related macular degeneration [28], cataract [29], uveitis [30], retinopathy of prematurity [31], keratitis [32] and glaucoma [4, 33]. The quantification of oxidative stress has therefore gained importance over the past few years.

There are many different ways of measuring oxidative stress, such as by quantifying the amount of anti-oxidants or by indirectly measuring the amount of oxidation of certain molecules (*e.g.* lipids) [34, 35]. We will focus on the comet assay methodology, also known as single-cell gel electrophoresis. Although this method reliably and reproducibly quantifies DNA breaks [3, 36], it is not completely standardized. For this reason, different laboratories use slightly different approaches: whereas some prefer to cryopreserve leukocytes prior to comet assay analysis, others perform comet assay on freshly prepared cells. We questioned whether the use of cryopreserved leukocytes -rather than freshly prepared leukocytes- would alter the final outcomes of single-stranded (ss)-DNA breaks in comet assay. The purpose of this study was to investigate the outcomes of comet assay analysis using both freshly prepared and cryopreserved leukocytes.

MATERIALS AND METHODS

Subjects

Two groups of ten healthy age- and sex-matched volunteers were recruited after a notification from the University of Basel informed potential volunteers of the opportunity to participate in a scientific research project. Ethical approval was obtained from the local medical ethics committee, and written informed consent was received from all subjects before entry into the study. The study was designed and conducted in accordance with the tenets of the Declaration of Helsinki. Exclusion criteria for both groups included history of ocular or systemic disease, smoking, drug or alcohol abuse, trauma, infection or inflammation. Demographic data for the different groups of subjects are given in Table 1.

Study design

Comet assay was performed on the leukocytes of 20 healthy subjects on the same day. Blood samples (20 ml) anticoagulated with heparin were obtained by venipuncture from all volunteers. The protocols for the study groups were the same, except for an additional round of cryopreservation and thawing for one experimental group. In the first group of ten subjects (group 1), leukocytes were isolated from the whole blood and comet assay was performed directly on the freshly isolated leukocytes. In the second group of ten subjects (group 2), blood samples were taken the day before comet assay analysis and the leukocytes were isolated and cryopreserved for 24 h. Details of the individual steps in the methods used for the two groups are described below.

Isolation of leukocytes (performed in both groups)

Blood samples (20 ml) anticoagulated with heparin were obtained by venipuncture from the two groups of patients. The leukocytes were isolated using Ficoll-Histopaque gradients (Histopaque 1077; Sigma-Aldrich, St. Gallen, Switzerland), as previously described [37]. The leukocyte bands were removed from the interface between the plasma and the histopaque layers of each tube and collected into one 50-ml tube. The total volume was brought to 50 ml with cold Dulbecco's Modified Eagle Medium (DMEM; Gibco®, Invitrogen, Basel, Switzerland). The cell suspension was washed three times with DMEM, and the total number of cells was determined. Cells were finally suspended in phosphate buffered saline (PBS) and aliquoted into Eppendorf tubes at 10^7 cells/tube.

Freshly prepared leukocytes for direct comet assay analysis (group 1)

The freshly prepared leukocytes from the first group were used directly for gel-electrophoresis and quantification of DNA breaks as described below.

Cryopreservation and thawing of leukocytes (group 2)

Aliquots of the isolated leukocytes were cryopreserved in freezing medium containing 90% heat inactivated fetal calf serum (FCS) and 10% dimethylsulfoxide (DMSO). The leukocytes for cryopreservation were immediately transferred into 1-ml cryopreservation tubes (Greiner Bio-One; Frickenhausen, Germany) at a concentration of 3×10^6 in 1 ml of freezing medium and frozen at a cooling rate of approximately –1°C/min to –80°C using a standard cryopreservation container where cell tubes are surrounded by isopropyl alcohol

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(Nalgene; Roskilde, Denmark). The cells were stored at –80°C for 24 h. For further comet assay analysis, the cells were rapidly thawed in a water bath at 37°C for 20 min until detachment of cells became visible. The leukocytes were then centrifuged for 3 min (1500 rpm) at 4°C to remove the freezing mix, and the leukocyte pellet was gently resuspended in 0.4 ml of FCS. Aliquots of this lymphocyte suspension were used for subsequent comet assay analysis.

Gel electrophoresis (performed in both groups)

Gel electrophoresis separates damaged DNA from undamaged DNA. The cells under study were embedded in agarose gel on a slide and subjected to lysis followed by electrophoresis under specific conditions. Electrophoresis was performed for 20 min at 300 mA and 25 V. To detect DNA after electrophoresis, the slides were stained with cyber-green and examined by fluorescence microscopy equipped with a personal computer-based analysis system (Kinetic Imaging; Nikon, Zurich, Switzerland), which enables quantification of DNA damage. Cells containing damaged DNA have the appearance of a comet with a bright head (undamaged) and tail (damaged; see Fig.1, arrow).

Quantification of DNA damage (performed in both groups)

It is recommended by the manufacturers of the comet microscope and imaging software (Nikon, Zurich, Switzerland) that 50 cells on each slide are chosen at random for the quantification of DNA damage using the computer software. Tail moment is defined as the product of the tail length and the tail DNA percentage of the total DNA:

Tail moment = tail length x tail DNA / 100

In addition, a function known as olive tail moment was evaluated. Olive tail moment represents the product of the distance between the centers of the mass of the head and tail regions and the tail DNA percentage of the total DNA:

Olive moment = (tail mean – head mean) x tail DNA / 100

Tail moment and olive tail moment are calculated by the computer software system as an average of the 50 cells selected for measurement. All the comets were quantified by a single observer.

Table 1. The demographic results for the two groups of subjects

	Age (Mean ± SD)	sex (m/f)
Group 1	49.5(14.5)	5/5
Group 2	50.5(14.22)	5/5
P value	0.88 (t-test)	1 (Fisher's exact test)

Comet assay analysis of ss-DNA breaks was performed on freshly prepared leukocytes in group 1 and on cryopreserved leukocytes in group 2

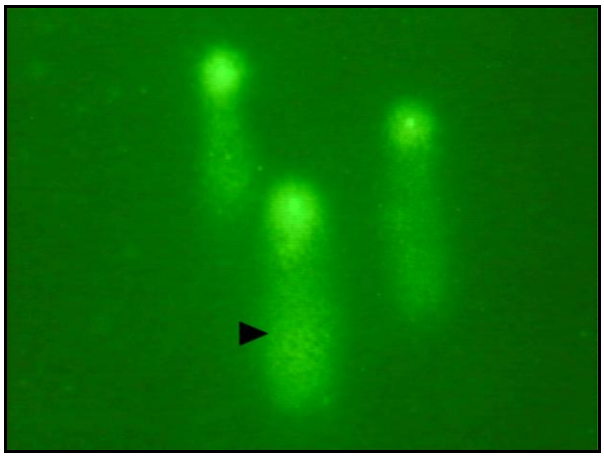

Figure 1. The cells analysed by comet assay analysis [38]. Each spot represents the DNA of an individual cell. Intact DNA is a large molecule that does not migrate much in the electrophoretic field. The less-bright green 'comet- shaped' area adjacent to the nucleus (arrow) represents DNA breaks that are small enough to move in the gel.

Statistical Analysis

The statistical evaluation was done in two steps: first, descriptive statistics, and then a test-statistical analysis. The test-statistics was done with both the parameter tail moment and the olive tail moment. As both parameters were zero-inflated (had many zeros), their distributions were heavy-tailed. To overcome this problem, the fractions of non-zero values compared to the total number of observations were counted for each subject. These fractions were approximately normally distributed. To compare the results of group 1 (freshly prepared leukocytes) to group 2 (cryopreserved leukocytes), Welch Two-Sample tests were performed. In comparison to the t-test, the Welch test allows for different standard deviations for each study group. Results of the tests are presented as differences of means between both study groups with corresponding 95% confidence intervals and P values. A P value less than 0.05 is considered significant. All evaluations were performed using the statistical package R version 2.8.1.

RESULTS

Demographic results for the two groups of subjects are presented in Table 1. The descriptive statistics for tail moment and olive moment for the two groups are shown in Table 2. Group 2 (cryopreservation of leukocytes) induced a significantly higher amount of single-stranded DNA damage in the circulating leukocytes than group 1 (freshly prepared leukocytes): tail moment 3.28 AU in comparison to 0.38 AU, and olive moment 1.89 AU in comparison to 0.25 AU, respectively; both $P < 0.01$ (Tables 2 and 3).

Table 2. The descriptive statistics of the tail moment and the olive moment in the two groups

	Mean	Median	SD	IQR	Minimum	Maximum	N
Tail moment							
Freshly prepared (group 1)	0.38	0	2.71	0	0	31.18	200
Cryopreserved (group 2)	3.28	0	15.32	0.11	0	197.88	436
All	2.37	0	12.84	0.03	0	197.88	636
Olive moment							
Freshly prepared (group 1)	0.25	0	1.54	0	0	17.31	200
Cryopreserved (group 2)	1.89	0.03	8.31	0.2	0	113.93	436
All	1.37	0	6.98	0.12	0	113.93	636

IQR = interquartile range; SD = standard deviation

Table 3. Differences between the means of the independent study groups

	Tail moment	Olive moment
Differences of means of the independent groups (group 2 *vs* group 1)	0.33	0.42
95% confidence interval	0.15 - 0.52	0.32 - 0.64
P value	0.003	< 0.001

The fraction of ss-DNA breaks comparing the results in groups 1 and 2 are shown by the parameters tail moment (Fig.2) and olive moment (Fig.3). As shown in both figures, the inter-individual difference in group 2 (cryopreserved leukocytes) was markedly higher than in group 1 (freshly prepared leukocytes).

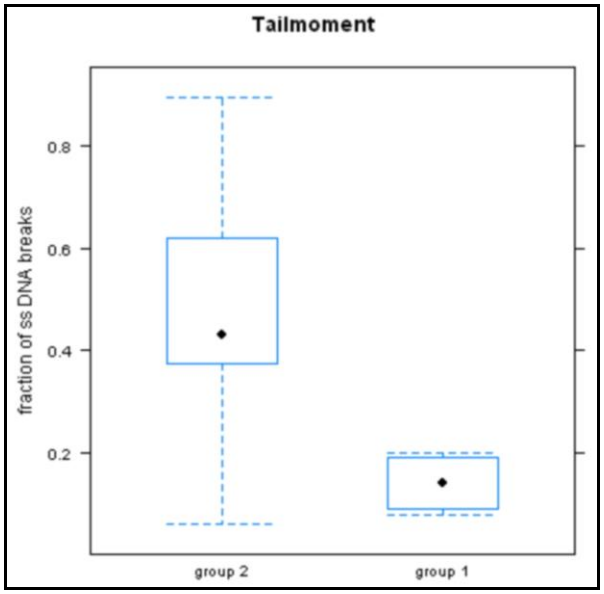

Figure 2. The fraction of ss-DNA breaks as measured by the parameter tail moment

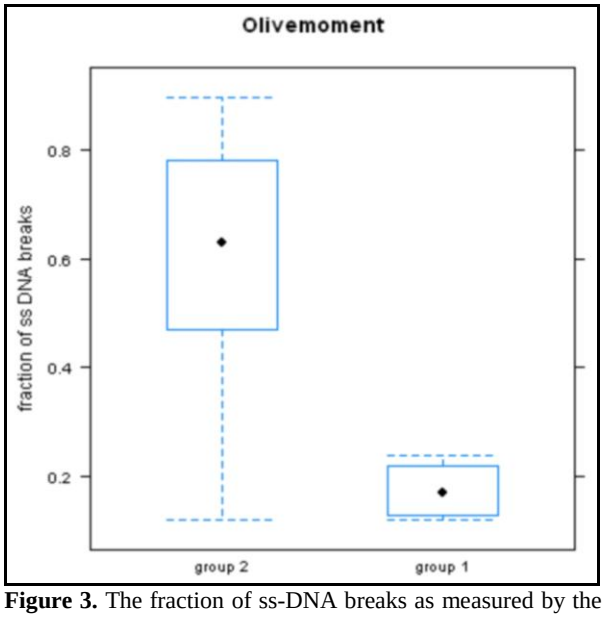

Figure 3. The fraction of ss-DNA breaks as measured by the parameter olive moment

DISCUSSION

Over the past twenty years, the comet assay has developed into an internationally recognized method for the detection of DNA breaks in various cell types [38, 39], particularly in leukocytes. The assay is considered to have high potential as a reliable method for detecting DNA breaks [40]. Although the method renders reproducible results, a number of steps in the method rely heavily on the skill of the individual examiner performing the assay. One of the factors that may influence the outcome of the assay is whether or not the leukocytes are cryopreserved. The purpose of our study was to evaluate this factor.

Leukocytes are cryopreserved using slightly different techniques in different laboratories [41-44]. Generally, cell damage in the freeze-thaw processes is either caused by extensive cell dehydration, by intracellular ice crystallization or a combination of both [45, 46]. The cooling rate is a critical step during freezing [47]. If cooling is sufficiently slow, the cell loses water rapidly enough by exosmosis to concentrate the intracellular solutes sufficiently to eliminate

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supercooling and maintain the chemical potential of intracellular water in equilibrium with that of extracellular water. The result is that the cell dehydrates [48] and does not freeze intracellularly. However, if the cell is cooled too rapidly, it is not able to lose water fast enough to maintain equilibrium; it becomes increasingly supercooled and eventually attains equilibrium by freezing intracellularly [49]. Intracellular ice formation leads to cell death. Leukocyte cryopreservation is usually performed using standard cryopreservation containers, where cell tubes are surrounded by isopropyl alcohol and simply placed inside a –80°C freezer (approximate cooling rate of –1°C/min) [50]. This was the cooling technique that we applied in our research. Perhaps a safer technique for cryopreservation is using computer-assisted controlled-rate freezers, which are thought to minimize the effects of both ice crystallization and cell dehydration [51].

Another important step in cryopreservation is the choice of the cyroprotectant added. The general properties required for cryoprotectant compounds are that they have low molecular weight, are nontoxic and are inexpensive [52, 53]. Cryoprotectants are divided into two main classes: intracellular agents which penetrate inside the cells, preventing ice crystal formation and membrane rupture (*e.g.* DMSO) [54], and extracellular compounds (*e.g.* sucrose, dextrose) [55] that do not penetrate the cell membrane and act by reducing the hyperosmotic effect present in the freezing procedure. The most commonly used cryoprotectant, DMSO, which was used in our study, provides a high rate of post-freezing cell survival, but it has been shown to have cytotoxic effects [56]. Disaccharides such as sucrose are not cytotoxic and have also been widely used as cryoprotectants [57]. The fact that they do not enter into cells is the main advantage, facilitating their removal after thawing. However, the precise mechanism by which disaccharides act to preserve biological systems during freezing is not well understood [58].

In conclusion, we found that cryopreservation of leukocytes evoked DNA damage. The use of cryopreserved leukocytes for comet assay analysis may therefore falsely alter the outcomes. Since the comet assay analysis method is gaining importance and many new users each year, we recommend that researchers either not use cryopreserved leukocytes for the assay, use freshly prepared leukocytes instead or be aware of the challenges involved.

REFERENCES

1. Ostling O, Johanson KJ. Microelectrophoretic study of radiation-induced DNA damages in individual mammalian cells. Biochem Biophys Res Commun 1984; 123:291-8.
2. Phillips JL, Singh NP, Lai H. Electromagnetic fields and DNA damage. Pathophysiology 2009; 16(2-3):79-88.
3. Dhawan A, Bajpayee M, Parmar D. Comet assay: a reliable tool for the assessment of DNA damage in different models. Cell Biol Toxicol 2009; 25:5-32.
4. Mozaffarieh M, Schoetzau A, Sauter M, Grieshaber M, Orgul S, Golubnitschaja O, et al. Comet assay analysis of single-stranded DNA breaks in circulating leukocytes of glaucoma patients. Mol Vis 2008; 14:1584-8.
5. Mozaffarieh M, Schotzau A, Josifova T, Flammer J. The effect of ranibizumab versus photodynamic therapy on DNA damage in patients with exudative macular degeneration. Mol Vis 2009; 15:1194-9.
6. Mozaffarieh M, Konieczka K, Hauenstein D, Schoetzau A, Flammer J. Half a pack of cigarettes a day more than doubles DNA breaks in circulating leukocytes. Tob Induc Dis 2010; 8:14.
7. Finkel T. Oxidant signals and oxidative stress. Curr Opin Cell Biol 2003; 15:247-54.
8. Halliwell B. Tell me about free radicals, doctor: a review. J R Soc Med 1989; 82:747-52.
9. Halliwell B. Reactive oxygen species in living systems: source, biochemistry, and role in human disease. Am J Med 1991; 91:14-22S.
10. Halliwell B, Gutteridge JM. The antioxidants of human extracellular fluids. Arch Biochem Biophys 1990; 280:1-8.
11. Halliwell B. Antioxidant defence mechanisms: from the beginning to the end (of the beginning). Free Radic Res 1999; 31:261-72.
12. Halliwell B. Reactive species and antioxidants. Redox biology is a fundamental theme of aerobic life. Plant Physiol 2006; 141:312-22.
13. Arbillaga L, Azqueta A, Ezpeleta O, Lopez de CA. Oxidative DNA damage induced by Ochratoxin A in the HK-2 human kidney cell line: evidence of the relationship with cytotoxicity. Mutagenesis 2007; 22:35-42.
14. Mozaffarieh M, Flammer J. Ocular Blood Flow and Glaucomatous Optic Neuropathy, Springer, Berlin-Heidelberg, Germany, 2009.
15. Halliwell B. Oxidative stress and neurodegeneration: where are we now? J Neurochem 2006; 97:1634-58.
16. Izzotti A, Sacca SC, Cartiglia C, De FS. Oxidative deoxyribonucleic acid damage in the eyes of glaucoma patients. Am J Med 2003; 114:638-46.
17. Karageuzyan KG. Oxidative stress in the molecular mechanism of pathogenesis at different diseased states of organism in clinics and experiment. Curr Drug Targets Inflamm Allergy 2005; 4:85-98.
18. Luthra A, Gupta N, Kaufman PL, Weinreb RN, Yucel YH. Oxidative injury by peroxynitrite in neural and vascular tissue of the lateral geniculate nucleus in experimental glaucoma. Exp Eye Res 2005; 80:43-9.
19. Mozaffarieh M, Grieshaber MC, Flammer J. Oxygen and blood flow: players in the pathogenesis of glaucoma. Mol Vis 2008; 14:224-33.
20. Ciccone S, Maiani E, Bellusci G, Diederich M, Gonfloni S. Parkinson's disease: a complex interplay of mitochondrial DNA alterations and oxidative stress. Int J Mol Sci 2013; 14:2388-409.

21. Sochocka M, Koutsouraki ES, Gasiorowski K, Leszek J. Vascular oxidative stress and mitochondrial failure in the pathobiology of Alzheimer's disease: new approach to therapy. CNS Neurol Disord Drug Targets 2013.

22. Wang JQ, Chen Q, Wang X, Wang QC, Wang Y, Cheng HP, Guo C, Sun Q, Chen Q, Tang TS. Dysregulation of mitochondrial calcium signaling and superoxide flashes cause mitochondrial genomic DNA damage in Huntington disease. J Biol Chem 2013; 288:3070-84.

23. Takeda S, Hui SP, Fuda H, Jin S, Sakurai T, Ishii A, Mukasa K, Sueoka K, Chiba H. Evaluation of various electrode materials for detection of oxidized low-density lipoproteins. J Biomed Nanotechnol 2013; 9:303-6.

24. Margaritis M, Antonopoulos AS, Digby J, Lee R, Reilly S, Coutinho P, Shirodaria C, Sayeed R, Petrou M, De Silva R, Jalilzadeh S, Demosthenous M, Bakogiannis C, Tousoulis D, Stefanadis C, Choudhury RP, Casadei B, Channon KM, Antoniades C. Interactions between vascular wall and perivascular adipose tissue reveal novel roles for adiponectin in the regulation of eNOS function in human vessels. Circulation 2013; 127:2209-21.

25. Mizukami H, Saitoh S, Machii H, Yamada S, Hoshino Y, Misaka T, Ishigami A, Takeishi Y. Senescence marker protein-30 (SMP30) deficiency impairs myocardium-induced dilation of coronary arterioles associated with reactive oxygen species. Int J Mol Sci 2013; 14:9408-23.

26. Wahab R, Dwivedi S, Umar A, Singh S, Hwang IH, Shin HS, Musarrat J, Al-Khedhairy AA, Kim YS. ZnO nanoparticles induce oxidative stress in Cloudman S91 melanoma cancer cells. J Biomed Nanotechnol 2013; 9:441-9.

27. Gukovsky I, Li N, Todoric J, Gukovskaya A, Karin M. Inflammation, autophagy, and obesity: common features in the pathogenesis of pancreatitis and pancreatic cancer. Gastroenterology 2013; 144:1199-209.

28. Klettner A, Kauppinen A, Blasiak J, Roider J, Salminen A, Kaarniranta K. Cellular and molecular mechanisms of age-related macular degeneration: From impaired autophagy to neovascularization. Int J Biochem Cell Biol 2013; 45:1457-67.

29. Stewart DN, Lango J, Nambiar KP, Falso MJ, FitzGerald PG, Rocke DM, Hammock BD, Buchholz BA. Carbon turnover in the water-soluble protein of the adult human lens. Mol Vis 2013; 19:463-75.

30. Saraswathy S, Rao NA. Posttranslational modification of differentially expressed mitochondrial proteins in the retina during early experimental autoimmune uveitis. Mol Vis 2011; 17:1814-21.

31. Wang H, Zhang SX, Hartnett ME. Signaling pathways triggered by oxidative stress that mediate features of severe retinopathy of prematurity. JAMA Ophthalmol 2013; 131(1):80-85.

32. Ananthi S, Chitra T, Bini R, Prajna NV, Lalitha P, Dharmalingam K. Comparative analysis of the tear protein profile in mycotic keratitis patients. Mol Vis 2008; 14:500-7.

33. Mozaffarieh M, Flammer J. New insights in the pathogenesis and treatment of normal tension glaucoma. Curr Opin Pharmacol 2012.

34. Chan SW, Chevalier S, Aprikian A, Chen JZ. Simultaneous quantification of mitochondrial DNA damage and copy number in circulating blood: a sensitive approach to systemic oxidative stress. Biomed Res Int 2013; 2013:157547.

35. Sanchez-Quesada JL, Perez A. Modified lipoproteins as biomarkers of cardiovascular risk in diabetes mellitus. Endocrinol Nutr 2013; pii:S1575-0922(13)00045-4.

36. Collins A, Dusinska M, Franklin M, Somorovska M, Petrovska H, Duthie S, Fillion L, Panayiotidis M, Raslova K, Vaughan N. Comet assay in human biomonitoring studies: reliability, validation, and applications. Environ Mol Mutagen 1997; 30:139-46.

37. Moenkemann H, Flammer J, Wunderlich K, Breipohl W, Schild HH, Golubnitschaja O. Increased DNA breaks and up-regulation of both G(1) and G(2) checkpoint genes p21(WAF1/CIP1) and 14-3-3 sigma in circulating leukocytes of glaucoma patients and vasospastic individuals. Amino Acids 2005; 28:199-205.

38. Olive PL. DNA damage and repair in individual cells: applications of the comet assay in radiobiology. Int J Radiat Biol 1999; 75:395-405.

39. Collins AR. The comet assay for DNA damage and repair: principles, applications, and limitations. Mol Biotechnol 2004; 26:249-61.

40. Moller P, Moller L, Godschalk RW, Jones GD. Assessment and reduction of comet assay variation in relation to DNA damage: studies from the European Comet Assay Validation Group. Mutagenesis 2010; 25:109-11.

41. Kwok A, Lyons BP, Hodges NJ, Bean TP. Cryopreservation and storage of mussel (*Mytilus* spp.) haemocytes for latent analysis by the Comet assay. Mutat Res 2013; 750:86-91.

42. Kong D, Shang H, Guo K, Liu Y, Zhang J, Wei H. A study on optimizing the cryopreservation methods for Bama miniature pig semen. Exp Anim 2012; 61:533-42.

43. Smagur A, Mitrus I, Giebel S, Sadus-Wojciechowska M, Najda J, Kruzel T, Czerw T, Gliwinska J, Prokop M, Glowala-Kosinska M, Chwieduk A, Holowiecki J. Impact of different dimethyl sulphoxide concentrations on cell recovery, viability and clonogenic potential of cryopreserved peripheral blood hematopoietic stem and progenitor cells. Vox Sang 2012; doi: 10.1111/j.1423-0410.2012.01657.x.

44. Pegg DE. Principles of cryopreservation. Methods Mol Biol 2007; 368:39-57.

45. Korpan NN, Hochwarter G, Sellner F. Cryoscience and cryomedicine: new mechanisms of biological tissue injury following low temperature exposure. Experimental study. Klin Khir 2009; 7-8:80-85.

46. Kim S, Agca C, Agca Y. Changes in rat spermatozoa function after cooling, cryopreservation and centrifugation processes. Cryobiology 2012; 65:215-23.

47. Janz FL, Debes AA, Cavaglieri RC, Duarte SA, Romao CM, Moron AF, Zugaib M, Bydlowski SP. Evaluation of distinct freezing methods and cryoprotectants for human amniotic fluid stem cells cryopreservation. J Biomed Biotechnol 2012; 2012:649353.

48. Oldenhof H, Friedel K, Akhoondi M, Gojowsky M, Wolkers WF, Sieme H. Membrane phase behavior during cooling of stallion sperm and its correlation with freezability. Mol Membr Biol 2012; 29:95-106.

49. Mazur P, Koshimoto C. Is intracellular ice formation the cause of death of mouse sperm frozen at high cooling rates? Biol Reprod 2002; 66:1485-90.

50. Recio L, Kissling GE, Hobbs CA, Witt KL. Comparison of Comet assay dose-response for ethyl methanesulfonate using freshly prepared versus cryopreserved tissues. Environ Mol Mutagen 2012; 53:101-13.

51. Buhl T, Legler TJ, Rosenberger A, Schardt A, Schon MP, Haenssle HA. Controlled-rate freezer cryopreservation of highly concentrated peripheral blood mononuclear cells results in higher cell yields and superior autologous T-cell stimulation for dendritic cell-based immunotherapy. Cancer Immunol Immunother 2012; 61:2021-31.

 DOI 10.5455/oams.050513.or.041

52. Johal AR, Schuman B, Alfaro JA, Borisova S, Seto NO, Evans SV. Sequence-dependent effects of cryoprotectants on the active sites of the human ABO(H) blood group A and B glycosyltransferases. Acta Crystallogr D Biol Crystallogr 2012; 68:268-76.

53. Rahman SM, Majhi SK, Suzuki T, Matsukawa S, Strussmann CA, Takai R. Suitability of cryoprotectants and impregnation protocols for embryos of Japanese whiting Sillago japonica. Cryobiology 2008; 57:170-4.

54. Pinisetty D, Alapati R, Devireddy RV. A molecular dynamics study of DMPC lipid bilayers interacting with dimethylsulfoxide-water mixtures. J Membr Biol 2012; 245:807-14.

55. Fonte P, Soares S, Costa A, Andrade JC, Seabra V, Reis S, et al. Effect of cryoprotectants on the porosity and stability of insulin-loaded PLGA nanoparticles after freeze-drying. Biomatter 2012; 2:329-39.

56. Oz ES, Aydemir E, Fiskin K. DMSO exhibits similar cytotoxicity effects to thalidomide in mouse breast cancer cells. Oncol Lett 2012; 3:927-9.

57. Li B, Tian F, Liu X, Zhao J, Zhang H, Chen W. Effects of cryoprotectants on viability of Lactobacillus reuteri CICC6226. Appl Microbiol Biotechnol 2011; 92:609-16.

58. Lillford PJ, Holt CB. *In vitro* uses of biological cryoprotectants. Philos Trans R Soc Lond B Biol Sci 2002; 357:945-51.

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