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Simple Cryoprotection and Cell Dissociation Techniques for Application of the Comet Assay to Fresh and Frozen Rat Tissues

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The single-cell gel electrophoresis (comet) assay has been widely used for genotoxicity studies in cell cultures, but its use in solid tissues is hindered by problems in isolation of cells and in cryopreservation techniques. Here, we used minced liver tissues from rats to compare a homogenization technique for isolation of nuclei with a collagenase digestion method (300 units/g liver at 37°C for 20 min) for isolation of intact cells for subsequent comet assay. We found that collagenase digestion was preferred to the homogenization technique in fresh tissues, but neither method prevented the extensive DNA damage caused by cryopreservation (−85°C for 72 h). To minimize this damage, minced liver (1.0 g) and kidney (0.5 g) tissues were added to 20 ml of pre-cooled 10% glycerol or 10% dimethylsulfoxide (DMSO). We showed that cryoprotection with DMSO (−85°C for 72 h and 3 weeks), and to a slightly lesser extent with glycerol (72 h), followed by collagenase digestion led to satisfactory recovery of liver cells with little or no DNA strand breakage. We then used DMSO as a cryoprotective agent to optimize the amount of collagenase and its incubation time in frozen liver and kidney tissues. We showed that the collagenase digestion at 150 units/g liver and 300 units/g kidney for 10 min produced highest cell numbers and minimal DNA strand breaks. We also validated these procedures by injection (i.p.) of rats with a known renal carcinogen, ferric nitrilotriacetate (Fe/NTA). We showed that Fe/NTA strongly induced DNA strand breaks in both rat liver and kidney, while no DNA strand breakage occurred in these tissues from the control rats. In addition, no significant differences in strand breaks were found between fresh tissues and tissues treated with DMSO during freezing at −85°C for 72 h. Thus, the cryoprotection and the cell dissociation techniques developed here are satisfactory for preparing both fresh and frozen tissues for comet assay. These simple techniques are expected to expand greatly the usefulness and efficacy of the assay.

Keywords: Comet assay; Tissue samples; Cryoprotection; Ferric nitrilotriacetate

Abbreviations: DMSO, dimethylsulfoxide; Na_2EDTA, disodium ethylenediamine tetraacetate; FE/NTA, ferric nitrilotriacetate; HBSS, Hank's balanced salt solution; LMP, low-melting-point; PBS, phosphate-buffered saline; TDNA, percentage of DNA in tail; TDx, tail distance; TMOM, Tail moment

INTRODUCTION

Oxidative damage to DNA is of great importance because of the growing recognition that such damage can both initiate and promote carcinogenesis.[1–5] Among various assays for measuring DNA damage, the single-cell gel electrophoresis (comet) assay is a sensitive and powerful method for determining DNA strand breakage,[6–9] and the results obtained from the assay have been shown to correlate with those from other genotoxicity assays.[10] Originally, the comet assay was carried out at neutral pH to measure double-stranded breakage of DNA, but was later modified to use alkaline pH (>12.6) so that the assay is capable of measuring both single- and double-stranded breaks as well as the alkaline-labile sites.[6,8,9] Applications of the comet assay have rapidly expanded to various fields.[7]

One limitation of the comet assay is that it has not been useful in solid tissues owing to difficulties in

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cell dissociation.[11] To solve this problem, some have employed enzymes[10,12,13] and others used mechanical techniques.[11,14–18] One study has compared the homogenization technique for isolating nuclei with the enzyme (collagenase plus trypsin) digestion method for isolating intact cells and concluded that the former method was quicker and simpler than the latter method.[17] The homogenization technique was later evaluated in a collaborative study by five laboratories, which showed that the technique is valid for fresh mouse organs in the *in vivo* comet assay.[11] However, we repeatedly found that the homogenization technique was rather unreliable and often led to high degree of DNA strand breakage. Another difficulty in using solid tissues for comet assay is that time is often a limiting factor when multiple samples have to be handled at the same time, and thus cryopreservation is desirable. However, freezing and thawing of solid tissues can produce artificially exaggerated levels of DNA strand breaks, as indicated by a study that utilized laser scanning microscopic analysis.[16] Therefore, the present study focused on fresh and frozen solid rat tissues to develop cell isolation and cryopreservation techniques suitable for the comet assay. We compared a homogenization technique with collagenase digestion of rat liver and kidney tissues before and after freezing at −85°C with or without glycerol or dimethylsulfoxide (DMSO), two commonly used cryoprotective agents.[19] In addition, we validated the standardized techniques by using fresh and frozen tissues from rats injected with ferric nitrilotriacetate (Fe/NTA), a renal and hepatic carcinogen.[20–22]

MATERIALS AND METHODS

Reagents

All chemicals used were of reagent or higher grade. Collagenase (EC 3.4.24.3) Type 1 was from Worthington Biochemical Corp. (Freehold, NJ). Glycerol was from Sigma Chemical (St Louis, MO), DMSO from Tedia (Fairfield, OH), and nitrilotriacetic acid (NTA) from Wako (Osaka, Japan). Ca^{2+}- and Mg^{2+}-free Hank's Balanced Salt Solution (1X HBSS, pH 7.4) consisted of 0.195 g KCl, 0.4 g KH_2PO_4, 8.0 g NaCl and 0.09 g $Na_2HPO_4 \cdot 7H_2O$ in 1000 ml of ddH_2O.

Preparation of Animal Tissues and Isolation of Cells from Fresh Liver and Kidney

Sprague–Dawley rats (200–300 g), purchased from the Animal Center of the National Science Council, were sacrificed by decapitation, and livers and kidneys were rinsed in cold phosphate-buffered saline (PBS, pH 7.4) and blotted dry on a filter paper. A portion of the fresh tissues was minced thoroughly on an aluminum foil in ice before homogenization or collagenase digestion. For homogenization studies, the minced liver was suspended at 1 ml/g of a cold homogenization buffer, pH 7.5, containing 0.075 M NaCl and 0.024 M Na_2EDTA[17] and homogenized gently (one stroke at the lowest speed) in ice using a Potter–Elvehjem glass homogenizer (GLAS-COL, GKH-GT, Apparatus, Terre Haute, IN). For enzymatic digestion, the minced tissues (1 g liver and 0.5 g kidney) were added to 10 ml of collagenase dissolved in 1X HBSS (Ca^{2+}- and Mg^{2+}-free), as modified from that reported previously.[17] The mixture was incubated with shaking at 37°C for 10, 20 or 30 min followed by a low centrifugation force (40*g*, 5 min) to remove undigested tissue debris. The supernatant was further centrifuged (700*g*, 10 min) to precipitate the cells, which were used for the comet assay immediately. Cell numbers and viability of isolated cells were determined using the Trypan blue dye, as described previously.[23]

Cryopreservation and Isolation of Cells from Frozen Liver and Kidney

For studying freezing effects on comet assay, a portion of the liver (1.0 g) and kidney (0.5 g) tissue was thoroughly minced and added to 20 ml of cold PBS containing 10% glycerol or 10% DMSO. The mixture was transferred into a 50 ml plastic centrifuge tube with cap and then frozen at −85°C for 72 h. The tissues were thawed in a water bath (37°C, 2–3 min) before homogenization or collagenase digestion, as described above.

Comet Assay

Comet assay was adapted from the method of Singh *et al.*[6] After isolation, cells were suspended in low-melting-point (LMP) agarose in PBS at 37°C and pipetted (75 μl, containing approximately 2×10^5 cells) onto a frosted glass microscope slide pre-coated with a layer of 1% normal-melting-point agarose. After application of a third layer of 1% normal-melting-point agarose, the slides were immersed in cold-lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% sodium lauryl sarcosinate, 1% Triton X-100) for 1 h at 4°C. The slides were then placed in an electrophoresis tank, allowing the DNA to unwind for 15 min in the alkaline solution (300 mM NaOH and 1 mM EDTA). The electrophoresis was then performed at 300 mA for 20 min in the same alkaline solution at room temperature. The slides were then neutralized with 0.4 M Tris–HCl buffer (pH 7.4) and stained with ethidium bromide. The image was analyzed both

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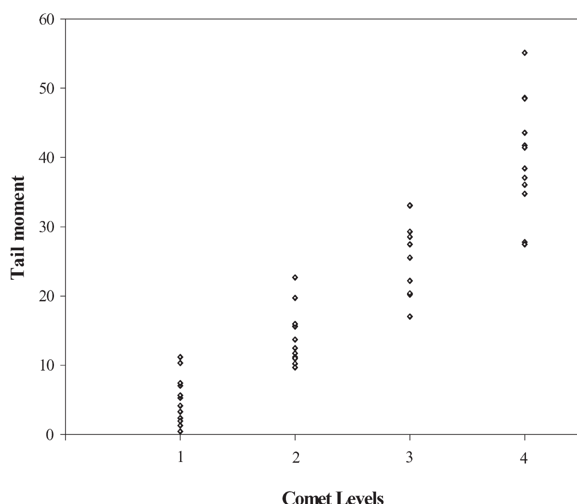

FIGURE 1 Correlation of tail moments and visual comet levels. The data are accumulated from the present study and a previous study[28] of the authors' laboratory.

visually as comet scores using the method of Collins *et al.*[24] and by computer using the Image Pro Plus software (Media Cybernetics, USA) as tail moment (TMOM) by the formula: TMOM = TDNA (i.e. percentage of DNA in tail) × TDx (i.e. tail distance). The tail moment is considered one of the best indices of comet formation by computerized analysis.[25–27] For visual analysis, 50 comets on each slide were scored according to tail intensity and given a value of 0 (no tail in any cells), 1 (low), 2 (medium), 3 (high), or 4 (maximal damage, i.e. all heads of comet are very small with most of the DNA in the tail). The visual scoring is subjective, but it has been shown to correlate with computer image analysis.[24] The relationship is confirmed by our cumulative data,[28] (and the present study) which show that class 1 damage is approximate equivalent to a tail moment of 1–10; class 2 is 11–20; class 3 is 21–35; and class 4 is 35–55 (Fig. 1).

Injection of Fe/NTA

Fe/NTA was prepared in a ratio of 1 to 4[21] by mixing 1 mM $Fe(NO_3)_3$ (dissolved in PBS) with 4 mM NTA (dissolved in PBS) just before injection. Sprague–Dawley rats (250–300 g) were fasted overnight and injected intraperitoneally with Fe/NTA (0.16 mmol/10 ml/kg body weight, equivalent to 9 mg Fe/10 ml/kg).[21] Control rats were injected with the same volume of PBS. Rats were sacrificed 30 min after injection by decapitation, and livers and kidneys were removed and used for comet assay either immediately or frozen with DMSO at −85°C for 72 h, as described above.

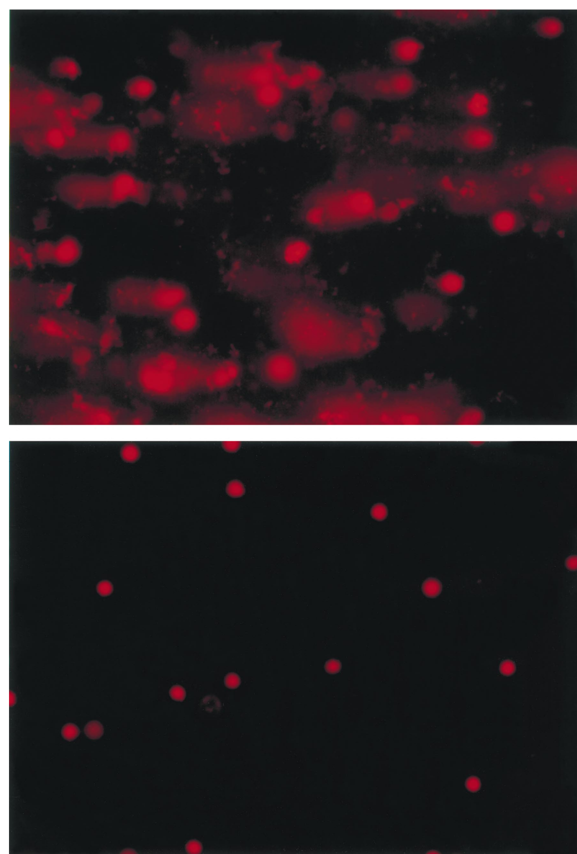

FIGURE 2 Representative comet images of DNA from fresh liver tissues obtained by the homogenization technique (upper) and the collagenase digestion method (lower). Fresh tissues (1.0 g liver and 0.5 g kidney) were minced thoroughly on ice before brief homogenization (at lowest speed, one stroke) or collagenase digestion (300 units/g liver at 37°C for 20 min). Nuclei or cells were collected by centrifugation and were used immediately for the comet assay.

Data Analysis

Values are expressed as means ± SD and analyzed using one-way ANOVA followed by Duncan's multiple range test for group mean comparisons.

RESULTS

Comparison of Homogenization Technique and Collagenase Digestion for Comet Assay

First, we used fresh-liver tissues to compare the homogenization technique for isolating nuclei with the collagenase digestion (300 units/g minced liver at 37°C for 20 min) method for isolating cells for subsequent comet assay. In five separate experiments, the homogenization technique resulted in relatively high DNA strand breakage (comet levels 1–2), with a tail moment of 10.1 and a large variation (SD = 7.7). By contrast, collagenase digestion produced intact cells with a survival rate of approximately 85% (data not shown) and with little or no

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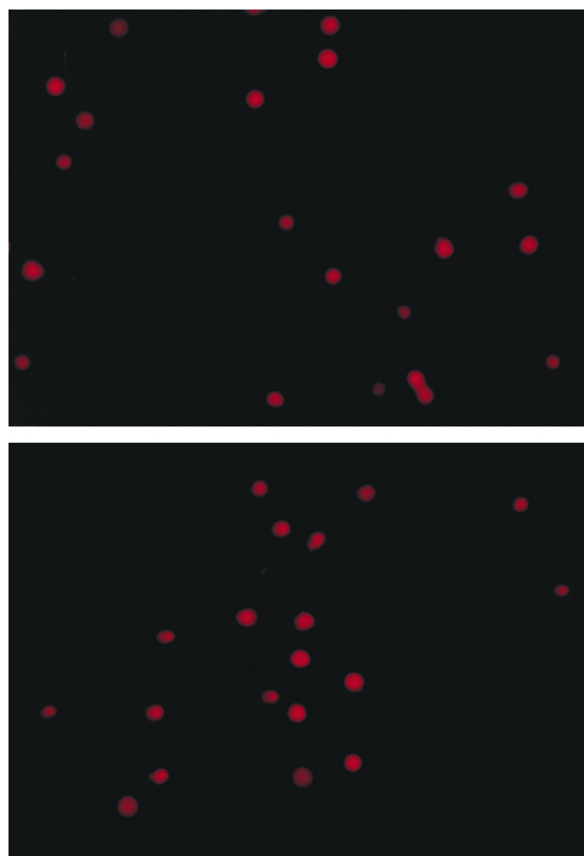

FIGURE 3 Representative comet images obtained from minced frozen liver tissues using pre-cooled DMSO (10% each in PBS) as a cryoprotective agent during freezing at −85°C for 3 days (upper photo) and 3 weeks (lower photo). The tissues were then thawed at 37°C for 2–3 min and the DMSO was decanted. Following collagenase digestion (300 units/g tissues) at 37°C for 20 min, the isolated cells were used for the comet assay.

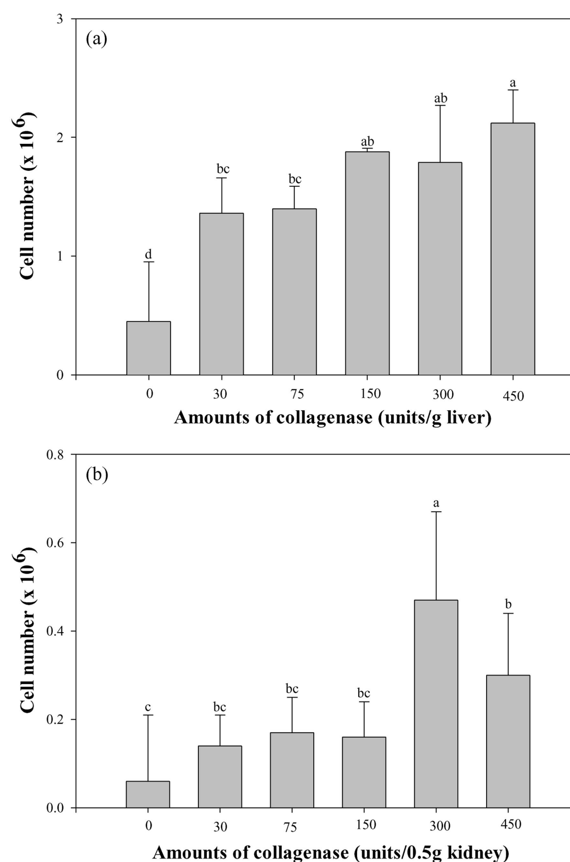

FIGURE 4 The concentration effect of collagenase on cell numbers (a) and extent of DNA strand breakage measured as tail moment (b) in tissues frozen with 10% DMSO at −85°C for 72 h. The incubation was carried out at 37°C for 20 min. Values (means ± SD of three separate experiments) sharing a common letter are not significantly different ($P > 0.05$).

DNA strand breaks (tail moment 1.1 ± 0.2) (data not shown). Figure 2 shows the typical comet image of DNA from fresh liver tissues obtained by the homogenization technique and the collagenase digestion. The nuclei isolated by the homogenization technique exhibited marked DNA damage (upper photo), while the cells isolated by collagenase digestion produced intact and well-separated DNA (lower photo). Thus, collagenase digestion is preferred to homogenization technique in preserving DNA in fresh tissues and was used in the following studies using frozen tissues.

Effects of Glycerol and DMSO During Cryopreservation Followed by Collagenase Digestion on Comet Assay

When tissues (minced or not minced) were frozen at −85°C for 72 h followed by collagenase digestion, extensive DNA strand breaks still occurred in the comet assay (data not shown), indicating that the freezing and the thawing cause considerable damage to DNA. To minimize the damage to DNA, a cold glycerol or DMSO solution (10% each in PBS) was added to the minced liver or kidney tissues during freezing (−85°C, 72 h). After thawing at 37°C for 2–3 min, the cells were isolated by collagenase digestion (300 units/g tissues) at 37°C for 20 min. The comet image shows that the DNA from frozen liver tissues treated with DMSO (upper photo, Fig. 3) was intact, with little or no strand breakage. Glycerol treatment also protected the DNA against damage during cryopreservation (photo not shown), although the tail moment of DNA (1.8 ± 0.1) was slightly higher than that of DMSO (1.4 ± 0.1). Using DMSO as the protective agent, we froze the minced liver at −85°C for 3 weeks and found essentially no increase in DNA strand breakage (lower photo, Fig. 3).

We also studied the dose effect of collagenase on the numbers of cells isolated from frozen tissues with 10% DMSO as cryoprotective agent. Figure 4a shows that the cell numbers increased with increasing amounts of collagenase per gram of

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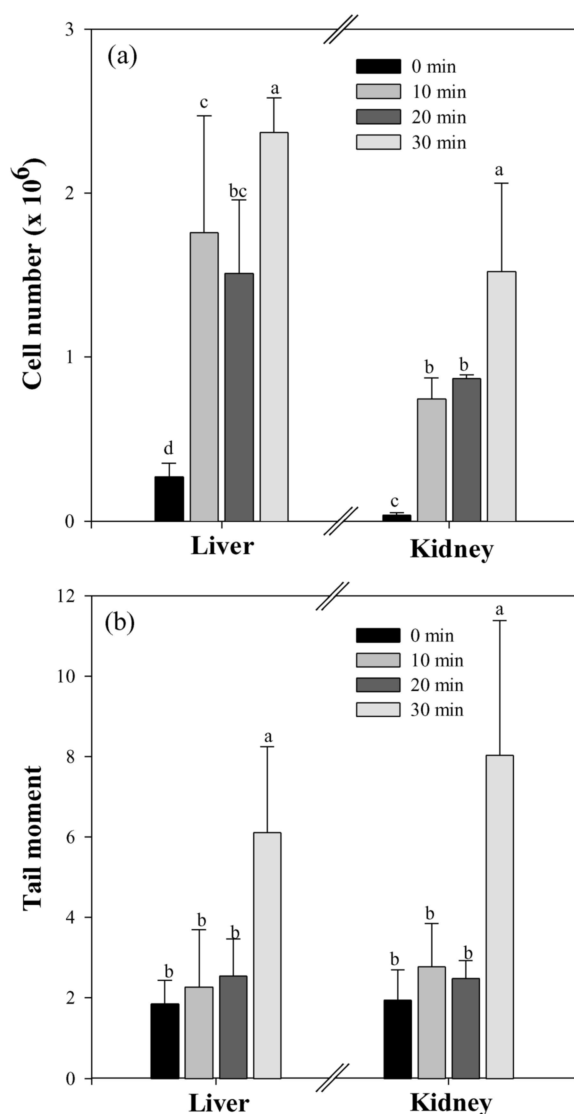

FIGURE 5 Time course of collagenase digestion at 37°C on cell numbers (a) and the extent of DNA breakage measured as tail moment (b) in tissues frozen with 10% DMSO at −85°C for 72 h. The amount of collagenase used in liver and kidney tissues were 150 and 300 units/g tissue, respectively. Values (means ± SD of three separate experiments) within each tissue sharing a common letter are not significantly different ($P > 0.05$).

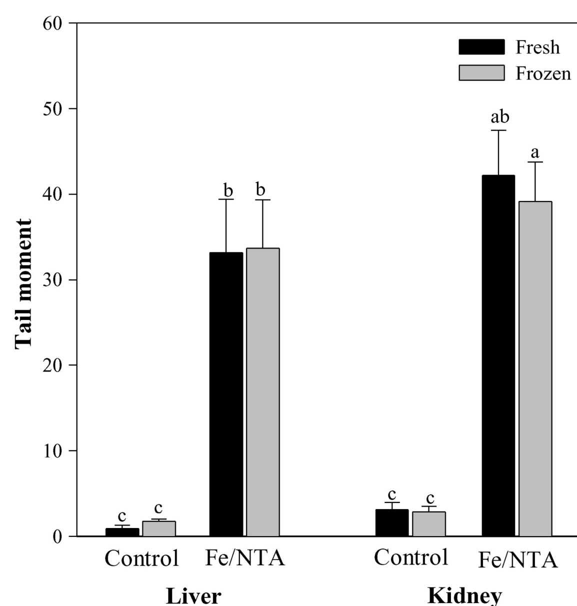

FIGURE 6 Effects of ferric nitrilotriacetate (Fe/NTA) injection (0.16 mmol/10 ml/kg body wt., i.p.) to rats on DNA strand breaks in fresh and frozen liver and kidney tissues using the recommended procedures for cryoprotection with DMSO at −85°C for 72 h and cell dissociation with collagenase (300 units/g for liver and 150 units/g for kidney) at 37°C for 10 min. Control rats were injected with the solvent PBS. Values (means ± SD, $n = 4$–5 rats) sharing a common letter are not significantly different ($P > 0.05$).

liver and reached a plateau (approximately 1.7×10^6 cells/g liver) at 150 units collagenase/g tissue. In frozen kidney, the dose-response effect of collagenase was less clear, with the highest cell number (4.6×10^5/0.5 g kidney) achieved at 300 units of collagenase/g tissue, while 450 units of collagenase led to significantly fewer cells than 300 units. We then studied the time course of collagenase digestion of tissues frozen at −85°C for 72 h with 10% DMSO as cryoprotective agent using 150 units/g liver and 300 units/0.5 g kidney. The results show that the cell numbers of both liver and kidney were significantly higher at 30 min than at 10 and 20 min of collagenase digestion, with the latter two time points not significantly different (Fig. 5a). However, the 30-min digestion time led to significantly higher tail moments in both frozen liver and kidney tissues (Fig. 5b). Thus, a 10-min collagenase digestion was considered appropriate for both frozen liver and kidney tissues before the comet assay.

Recommended Techniques for Cell Dissociation and Cryoprotection of Liver and Kidney Tissues

For fresh tissues, a portion of liver (1.0 g) or kidney (0.5 g) was minced thoroughly in aluminum foil on ice, transferred into a 50 ml flask with 10 ml of a collagenase solution (15 units/ml for liver and 30 units/ml for kidney). After incubation at 37°C with shaking for 10 min, the mixture was centrifuged at low speed (40*g*, 5 min at 4°C) to remove undigested tissue debris. The supernatant was further centrifuged (700*g*, 10 min at 4°C) to precipitate the cells, which were washed in 1 ml of PBS and transferred to an Eppendorf tube followed by another centrifugation (700*g*, 5 min at 4°C. The supernatant was decanted, and the cells were suspended in LMP agarose (approximately 1 and 0.5 ml for liver and kidney tissues, respectively) and used immediately for the comet assay, as described above.

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For cryopreservation, liver (1.0 g) and kidney (0.5 g) tissues were minced thoroughly on aluminum foil in ice, transferred into a 50 ml plastic centrifuge tube with cap, and added 20 ml of cold PBS containing 10% DMSO (both PBS and DMSO were pre-cooled in ice before use). After freezing at −85°C for 72 h (or longer period), the mixture was thawed in a 37°C water bath for 2–3 min to melt the cryopreserving liquid, which was then decanted. The tissue was transferred into a 50-ml flask with 10 ml of collagenase solution, as described in the procedures for fresh tissues.

Application of the Standard Procedures to Rats Injected With Fe/NTA

To validate the techniques recommended above, we injected rats with Fe/NTA and measured DNA strand breaks in liver and kidney by the comet assay. The results show that Fe/NTA resulted in strong strand breakage of DNA in both rat liver and kidney, while the tissues from control rats (injected with PBS to replace Fe/NTA) showed no DNA strand breaks by the same recommended techniques (Fig. 6). In addition, there were no significant differences between fresh tissues and frozen tissues.

DISCUSSION

The comet assay is a sensitive and powerful tool for genotoxicity studies, but its use in fresh and frozen solid tissues is not well characterized. Here, we have standardized procedures for preventing DNA damage during cryopreservation and isolation of cells in both fresh and frozen rat liver and kidney tissues for subsequent comet assay. The standardized procedures employed collagenase digestion for isolation of cells, rather than the homogenization technique for isolating nuclei recommended previously,[11,17,18] because the latter technique led to significant DNA strand breakage in both fresh and frozen tissues. For frozen tissues, inclusion of 10% DMSO in minced tissues during cryopreservation before cell dissociation by collagenase digestion was necessary to prevent DNA strand breakage. The recommended procedures produce approximately $1.5–1.8 \times 10^6$ cells/g liver and $5–8 \times 10^5$ cells/0.5 g kidney, which are sufficient for preparing seven and three comet slides for liver and kidney, respectively (we commonly used approximately 2×10^5 cells on one slide).

Collagenase perfused to rat liver for isolation of hepatocytes was reported to cause DNA damage measured using the alkaline elution technique, and the effect is related to perfusion time.[29] Although we used *in vitro* incubation of collagenase, rather than the *in situ* perfusion technique, we also observed that collagenase increased DNA strand breaks in liver and kidney tissues when the incubation time reached 30 min at 37°C. However, we showed that the collagenase digestion conditions could be easily controlled to avoid such damage. It is worth mentioning that, although several groups of workers have used and advocated the homogenization technique in solid tissues,[11,17,18] no comet images with good success by this technique have, to our knowledge, been presented to date.

Cryopreservation of animal tissues is often necessary in various types of biological studies. However, we found that the simple cryopreservation led to extensive DNA strand breaks in both rat liver and kidney tissues, possibly due to freezing and thawing. Our findings are in accord with those reported by Fairbairn *et al*.[16] that cryopreservation of mouse tissues produces artificially elevated levels of DNA damage. We, therefore, added DMSO to minced tissues before freezing. A concentration of 5–10% DMSO is commonly recommended for cell preservation since higher concentrations may lead to cell damage.[30] Here, a 10% DMSO in PBS was found to prevent artificial DNA strand breaks in liver and kidney tissues during preservation. It is important to note that the DMSO must be pre-cooled and mixed with cold PBS before adding to the minced tissues to avoid possible damage to cells.[30] Another point to note is that DMSO had no protection when added to tissues that were not minced before freezing, possibly due to difficulties of DMSO in penetration into hard tissues.

In order to validate these procedures, we applied the collagenase digestion method and cryoprotective techniques to rats injected with Fe/NTA, a hepatic and renal carcinogen.[20–22] As expected, Fe/NTA injection leads to strong DNA damage in the kidney and slightly less damage in the liver, while the control rats had essentially no DNA strand breaks in both fresh and frozen kidney and liver tissues. In addition, there were no significant differences in DNA strand breaks between fresh tissues and frozen tissues, indicating that DMSO protects against DNA strand breakage during cryopreservation.

In summary, we have developed simple cryoprotection and cell dissociation techniques suitable for applying the comet assay to both fresh and frozen animal tissues. With these techniques, the comet assay may now be extended to various solid tissues including normal and tumor tissues. Thus, it is possible to conduct *in vivo* genotoxicity studies on various agents and to readily measure DNA strand breaks in the tissues using the comet assay. For instance, liver and kidney tissues can now be conveniently used for measuring DNA strand breaks by simple collagenase digestion to replace the laborious *in situ* perfusion technique to isolate hepatocytes. In addition, because tissues can now

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be frozen for at least a few weeks before the comet assay, time can be better used and efficiency can be gained in various research works.

Acknowledgements

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References

[1] Ames, B.N. and Gold, L.S. (1991) "Endogenous mutagens and the causes of aging and cancer", *Mutat. Res.* **250**, 3–16.
[2] Ames, B.N., Gold, L.S. and Willet, W.C. (1995) "The causes and prevention of cancer", *Proc. Natl Acad. Sci. USA* **92**, 5258–5265.
[3] Cerutti, P., Larsson, R., Krupitza, G., Muehlmatter, D., Crawford, D. and Amstad, P. (1989) "Pathophysiological mechanisms of active oxygen", *Mutat. Res.* **214**, 81–88.
[4] Floyd, R.A. (1990) "The role of 8-deoxyguanine in carcinogenesis", *Carcinogenesis* **11**, 1447–1450.
[5] Lindahl, T. and Wood, R.D. (1999) "Quality control by DNA repair", *Science* **286**, 1897–1905.
[6] Singh, N.P., McCoy, M.T., Tice, R.R. and Schneider, E.L.A. (1988) "A simple technique for quantitation of low levels of DNA damage in individual cells", *Exp. Cell Res.* **175**, 184–191.
[7] Fairbairn, D.W., Olive, P.L. and O'Neill, K.L. (1995) "The comet assay: a comprehensive review", *Mutat. Res.* **339**, 37–59.
[8] Collins, A.R., Dobson, V.L., Dusinska, M., Kennedy, G. and Stetina, R. (1997) "The comet assay: what can it really tell us?", *Mutat. Res.* **375**, 183–193.
[9] Halliwell, B. and Gutteridge, J.M.C. (1999) Free Radicals in Biology and Medicine, 3rd ed. (Oxford University Press, New York), Figure 4.8, pp 268–269.
[10] Monteith, D.K. and Vanstone, J. (1995) "Comparison of the microgel electrophoresis assay and other assays for genotoxicity in the detection of DNA damage", *Mutat. Res.* **345**, 97–103.
[11] Miyamae, Y., Yamamoto, M., Sasaki, Y.F., Kobayashi, H., Igarashi-Soga, M., Shimoi, K. and Hayashi, M. (1998) "Evaluation of a tissue homogenization technique that isolates nuclei for the *in vivo* single cell gel electrophoresis (comet) assay: a collaborative study by five laboratories", *Mutat. Res.* **418**, 131–140.
[12] Tice, R.R., Andrews, P.W. and Singh, N.P. (1990) "The single cell gel electrophoresis assay: a sensitive technique for evaluating intracellular differences in DNA damage and repair", *Basic Life Sci.* **53**, 291–301.
[13] Betti, C., Davini, T., Giannessi, L., Loprieno, N. and Barale, R. (1993) "Comparative studies on cytotoxic and genotoxic effects of two organic mercury compounds in lymphocytes and gastric mucosa cells of Sprague–Dawley rats", *Environ. Mol. Mutagen.* **22**, 172–180.
[14] Plappert, U., Barthel, E., Raddatz, K. and Seidel, H.J. (1994) "Early effects of benzene exposure in mice. Hematological versus genotoxic effects", *Arch. Toxicol.* **68**, 284–290.
[15] Sirota, N.P., Bezlepkin, V.G., Kuznetsova, E.A., Lomayeva, M.G., Milonova, I.M., Ravin, V.K., Gaziev, A.I. and Bradbury, R.J. (1996) "Modifying effect *in vivo* of interferon on induction and repair of lesions in DNA of lymphoid cells of gamma-irradiated mice", *Radiat. Res.* **146**, 100–105.
[16] Fairbairn, D.W., Reyes, W.A., Grigsby, R.V. and O'Neill, K. (1994) "Laser scanning microscopic analysis of DNA damage in frozen tissues", *Cancer Lett.* **76**, 127–132.
[17] Sasaki, Y.F., Tsuda, S., Izumiyama, F. and Nishidate, E. (1997) "Detection of chemically induced DNA lesions in multiple mouse organs (liver, spleen, kidney, and bone marrow) using the single cell gel electrophoresis (Comet) assay", *Mutat. Res.* **388**, 33–44.
[18] Sasaki, Y.F., Nishidate, E., Izumiyama, F., Matsusaka, N. and Tsuda, S. (1997) "Simple detection of chemical mutagens by the alkaline single-cell gel electrophoresis (Comet) assay in multiple mouse organs (liver, spleen, kidney, and bone marrow)", *Mutat. Res.* **391**, 215–231.
[19] Mazur, P. (1970) "Cryobiology: the freezing of biological systems", *Science* **168**, 939–949.
[20] Ebina, Y., Okada, S., Hamazaki, S., Ogino, F., Li, J.I. and Midorikawa, O. (1986) "Nephrotoxicity and renal cell carcinoma after use of iron- and aluminum nitrilotriacetate complexes in rats", *J. Natl Inst. Cancer* **76**, 107–113.
[21] Iqbal, M. and Arthur, M. (1998) "Attenuation of iron–nitrilotriacetate (Fe–NTA)-mediated renal oxidative stress, toxicity and hyperproliferative response by the prophylactic treatment of rats with garlic oil", *Food Chem. Toxicol.* **36**, 485–495.
[22] Iqbal, M., Giri, U. and Arthur, M. (1995) "Ferric nitrilotriacetate (Fe-NTA) is a potent hepatic tumor promoter and acts through the generation of oxidative stress", *Biochem. Biophys. Res. Commun.* **212**, 557–563.
[23] Phillips, H.J. (1973) "Dye exclusion tests for cell viability", In: Kruse, P.F. and Patterson, M.K., eds, Tissue Culture, Method and Applications (Academic Press, New York), pp 406–408.
[24] Collins, A.R., Ma, A-G. and Duthie, S.J. (1995) "The kinetics of repair of oxidative DNA damage (strand breaks and oxidized pyrimidines) in human cells", *Mutat. Res.* **336**, 69–77.
[25] Olive, P.L., Banath, J.P. and Durand, R.E. (1990) "Heterogeneity in radiation-induced DNA damage and repair in tumor and normal cells using the comet assay", *Radiat. Res.* **122**, 86–94.
[26] Mckelvey-Martin, V.J., Green, M.H.L., Schmezer, P., Pool-Zobel, B.L., De Meo, M.P. and Collins, A. (1993) "The single cell gel electrophoresis assay (comet assay): a European review", *Mutat. Res.* **288**, 47–63.
[27] Collins, A.R. (1992) "Workshop on single cell gel electrophoresis (the "comet" assay)", *Mutagenesis* **7**, 357–358.
[28] Yeh, S.-L. and Hu, M.-L. (2001) "Induction of DNA damage in human foreskin fibroblast Hs68 cells by oxidized β-carotene and lycopene", *Free Radic. Res.* **35**, 203–213.
[29] Cesarone, C.F., Fugassa, E., Gallo, G., Voci, A. and Orunesu, M. (1984) "Collagenase perfusion of rat liver induces DNA damage and DNA repair in hepatocytes", *Mutat. Res.* **141**, 113–116.
[30] Chen, C.-C. (1998) "The cryopreservation of animal cell lines", *Food Indust. (Taiwan)* **30**, 44–50.

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