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A Comparison of Hydrated versus Dehydrated Gels for Evaluation of Apoptosis in Comet Assay Slides

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Comet assay slides 에서 나타난 apoptosis 평가에서 함수 및 탈수 겔의 비교

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요 약 : Comet assay는 포유류 세포에서 DNA의 파괴를 측정하는데 있어 신속하고 단순하며 시각적이고 민감한 방법이다. Apoptosis에서는 세포핵의 광범위한 DNA의 붕괴가 일어나므로 comet assay는 종양세포에서 apoptosis가 발생되었는 가를 알아내는데 유용하다. 본 연구는 apoptosis 연구의 결과가 변화되지 않도록 comet assay slides를 좀 더 오래 보관할 수 있는 방법을 개발하고자 시행되었다. 개의 종양세포를 가지고 alkali comet assay를 끝낸 뒤 slides를 진공 건조기에서 건조시켰다. 건조된 slides는 건조후 3-4일, 1주, 2주, 3주, 4주 및 7주째에 건조기에서 꺼내서 증류수로 점적하여 10-20분간 침수시키고 현광현미경하에서 육안적으로 관찰하였다. 건조후 3-4일, 1주, 2주, 3주, 4주 및 7주의 slides에서 apoptosis 회복율(%)은 각각 98.1, 98.3, 99.4, 97.3, 80.0 및 35.2%이었다. 3주 이내의 slides에서는 대조군과 비교하여 apoptosis 회복율에서 차이가 없었으나 4주 이상의 slide에서는 대조군과 유의성 있는 차이를 보였다(P<0.05). 이러한 결과로 보아 comet slides를 건조후 침수시키는 방법을 이용하였을 때 apoptosis 평가에서 건조후 3주간까지는 처음의 결과와 차이가 없으며, 이 방법을 이용하여 comet slide의 좀 더 긴 기간의 보관과 보관후의 재평가에서 이용될수 있는 좋은 방법이 된다.

Key words : apoptosis, comet assay, hydrated gel, dehydrated gel

Introduction

The comet assay used for the direct visualization of DNA damage in individual cells was first introduced by Östling and Johanson in 1984[21]. It is also called the single cell gel assay (SCG) and microgel electrophoresis (MGE).

The basis of the method is that a broken DNA molecule can migrate more readily in an electric field than an intact molecule. When cells are embadded in agarose and subsequently lysed to remove protein, smaller DNA molecules are able to migrate away from the residual nucleus. When DNA is subsequently stained with flourescent dye, the cell resembles a comet with a head region containing un-

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damaged DNA and a tail containing the broken DNA. The amount of DNA to be able to migrate and, to a lesser extent, the distance of migration are indications of the number of strand breaks present in that cell[14].

In spite of a short developing history of comet assay, it is widely used in radiation biology[3,9,11,12,15,16,23], study of excisable DNA damage[2,5], DNA crosslinks relevant cytotoxic lesion produced by many chemotherapeutic agents[13], oxidative damage[1,6], genetic toxicology[4,8,7], and apoptosis[18,20,24]. Since apoptosis is characterized by extensive DNA cleavage, the comet assay can be useful in detecting apoptotic cells in tumor cells[18]. But comet slides shoud be counted within 2 days after procedure. For over 3 days, comet slides are no longer good to count and analysis for poor image. This has led to the need to develop a quality control procedure to aid in keeping the comet assay slides for some time without altering results of apoptosis research.

This paper demonstrated the ability of a drying method to detect apoptotic cells on comet assay slides by reconstitution with distilled water.

Materials and Methods

Materials

55 client-owned dogs with tumors were evaluated at Colorado State University (CSU). Each dog had been received a clinical evaluation, regional and thoracic radiographs, serum biochemistry profile, and a complete blood count as the initial evalution. Diagnosis of tumors was confirmed by histopathological findings. Before chemical and radiation theraphy, each tumor sample was obtained from biopsies and surgical resection during operation.

The comet assay (alkaline assay)[10]

A standard protocol for comet preparation and analysis was adopted. With tumor samples, a single cell suspension was made in 0.5-1 ml PBS (2×10^6 cells/ml). Agarose (1.5 ml) at 40°C was added to the 0.5 ml of cell suspension, and the solution was pippetted onto a poly-L-lysine treated slide. Eight slides were made for a tumor sample. After letting the slides gel on a cold surface for 1 minute, they were then immersed in a lysing solution consisting of 0.03 M NaOH, 1 M NaCl and 0.1% N-lauroysarcosine for 1 h in the dark at room temperature. To remove the salt, slides were rinsed 2 times for 20 minutes in 0.03 M NaOH, 2 mM EDTA. Horizontal gel electroporesis was performed in a fresh solution of 0.03 M NaOH, 2 mM EDTA at 0.5 V/cm for 25 minutes. After the slides were rinsed in distilled water for 10 minutes, DNA was stained by immersing slides in 2.5 μg/ml propidium iodide (PI) for 10 min. Apoptotic cells were detected "by eye" on slides using a flourescence microscope. Over 400 individual cells on a slide were analyzed for each sample.

Drying method

Except for 2 control slides, 6 slides were put in a vacuum dessicator. For short drying time of study, slides were taken out after 3-4 days of drying and reconstitued by dropping distilled water and allowed to wait for 10-20 minutes. For longer drying time of study, slides were taken out after 1, 2, 3 and 4 weeks of drying (a few slides were after 7 weeks of drying) and reconsituted with distilled water and counted "by eye" using a fluorescence mocroscope.

Data analysis

After counting the tumor cells, percent tumor apoptosis was calculated.

To compare the percent tumor apoptosis of each slide with that of control slides, we calculated the recovery rate (%) by dividing the percent tumor apoptosis of dried slides with that of control slides and timing 100. Statistic analysis was performed using paired T-test.

Results

After for 3-4 days, 1, 2, 3, 4, and 7 weeks of drying period, 2 slides were taken out from the vacuum dessicator and reconsituted with distilled water.

Most comets on reconsituted slides were good to be counted by eye. But as time went by, the images of comets worsened and sometimes slides needed

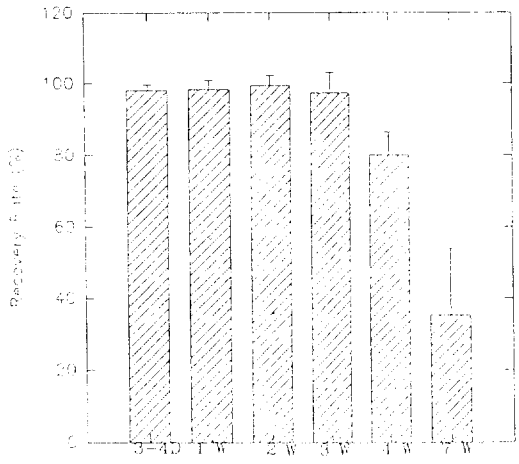

Fig 1. Appoptotic changes during dryign period in canine tumors (Mean, SEM).

more time (over 1 hour) for reconstitution compared to normal 10-20 min. of reconstitution time. After 7 weeks of drying, most comets were indistinguishable.

The recovery rates at 3-4 days, 1, 2, 3, 4, and 7 weeks were 98.1, 98.3, 99.4, 97.3, 80.0, and 35.2%, respectively (Fig. 1). There were no significant differences in recovery rates between control slides and 3-4 days, 1, 2, 3 weeks of drying slides, whereas there were significant differences between control slides and 4 and 7 weeks of drying slides ($P<0.05$, Fig. 1).

Discussion

After Östling and Hohanson first introduced the comet assay in 1984, subsequent changes to the original lysis, electrophoresis, and DNA staining protocol, and development of video image analysis methods have greatly improved accuracy, sensitivity, and speed of measurements[17,19,22,25]. This method has been widely used in radiation biology, studies of DNA damage, and toxicology. As the apoptosis is characterized by DNA damage in breakage, this comet assay can be used for apoptosis study with efficiency[18,20,24]. The extent of DNA damage in apoptotic cells is so great that they are easily distinguishable visually from undamaged cell.

In our pilot experiment, the comet assay seemed to be highly reproducible for apoptotic tumor cells on the same reconsituted slides. As our rusults show in this experiment, reconsituted slides were safe and reproducible within 3 weeks. In a few slides of 4 weeks of drying period, recovery rates were good, but most images were bad. In reconsitution with distilled water, some slides sometimes needed more time (over 1 hour) to be rehydrated, but in most cases 20 minutes were enough to reconsitute.

We can conclude that dehydration and reconstitution of comet slides for up to 3 weeks is a simple, safe and reproducible method without changing results for evaluation of apoptosis in tumor cells. This dehydration and reconstitution method may contribute to apoptosis study by saving the comet slides for 3 weeks without result differences and further usages for DNA damage-related researches.

Summary

The comet assay is a rapid, simple, visual and sensitive technique for measuring DNA breakage in individual mammalian cells. As apoptosis is characterized by extensive DNA cleavage, the comet assay can be used in detecting apoptoic cells in tumor cells. But comet slides shoud be counted within 2 days after prodedure. For over 3 days, comet slides are no longer good to be counted for poor image. This has led to the need to develop a quality control procedure to aid in keeping the comet assay slides without altering apoptotic change. With canine tumor cells, comet slides were made and slides were put in a vacuum dessicator. During 7 weeks of drying period, slides were taken out at 3-4 day, 1, 2, 3, 4, and 7 week intervals. Each slides was reconsituted by dropping distilled water and allowed to set for 10-20 minutes then apoptotic cells were counted "by eye" using a fluorescence microscope. The recovery rates at 3-4 days, 1, 2, 3, 4, and 7 weeks were 98.1, 98.3, 99.4, 97.3, 80.0, and 35.2 %, respectively. There were no significant differences in recovery rates between control slides and 3-4 days, 1, 2, 3 weeks of drying slides, whereas there were significant differences between control slides

and 4 and 7 weeks of drying slides ($P<0.05$). These results indicate that dehydration and reconsituting method of comet slides is good for apoptosis study up to 3 weeks post dehydration.

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