Current Topics in Toxicology
Vol. 16, 2020

Original Article

Evaluation of human genomic DNA damage caused by H_2O_2 *in vitro* using single-cell gel electrophoresis

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ABSTRACT

The comet assay or single-cell gel electrophoresis (SCGE) has been widely used over a decade as a simple, rapid, and sensitive way for determining deoxyribonucleic acid (DNA) strand breaks. The versatility of the SCGE assay is because of the fact that a wide range of fresh or frozen samples can be used, including peripheral blood, cultured cells, buccal mucosal cells, solid tumours, cancer cells, yeast cells, epithelial cells, bacteria etc. The most extensively used form of this method is the alkaline version as it detects a variety of damages in single cells, such as DNA double-strand breaks, single-strand breaks, alkali-labile sites and DNA-DNA/DNA-protein cross-linking. Thus, the human epithelial bladder cancer (RT112) cell line was incubated with hydrogen peroxide (H_2O_2), a DNA-damaging agent. The cells were embedded in a thin agarose gel on microscope slides. Cellular proteins were removed by lysing the cells, followed by unwinding the damaged DNA-forming comets using electrophoresis under alkaline conditions (pH >13). One hundred comet images per each concentration were captured by using a fluorescence microscope. There was a significant increase in DNA percentage in tail of the comets with a linear rising of the tail lengths. Additionally, there was a strong positive correlation between tail DNA percentage and the lengths of the tail comets ($p < 0.05$, $r^2 > 0.98$). Overall, the results of this study indicate that by increasing the DNA damage by H_2O_2, the intensity of the comet tails becomes higher, and the alkaline form of the comet assay is an effective method for measuring this damage.

KEYWORDS: comet assay, DNA damage, hydrogen peroxide, tail DNA percentage.

1. INTRODUCTION

It is undeniable that the individual genomic DNA is highly likely to be damaged not only by exogenous materials but also by exposure to endogenous processes [1]. For example, reaction oxygen species (ROS) are uninterruptedly formed in living cells and stem from chemical activities and other metabolic processes [2]. It has become widely accepted that H_2O_2 is an intracellular byproduct of oxidative metabolism, and it is catalysed into glutathione peroxidases (GPx), peroxiredoxins and water under normal physiological conditions [3]. As a result, the reducing agents like ascorbic acid have been shown to release free radicals in the cells during metabolism processes [4, 5]. However, if the competence of metabolism enzyme systems is inadequate, H_2O_2 may highly react with transition metals, such as iron and copper, to produce the highly reactive and damaging hydroxyl radical ($OH^{\bullet}$) [6]. Hydroxyl radical is considered as a free radical, and it is a notorious agent for single strand DNA (ssDNA) break formation [7]. Numerous studies have confirmed that this damage, which leads to genomic instability, brings about a number of diseases including cancers and arteriosclerosis [8, 9].

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Virtually all cellular molecules and biochemical structures are under threat by ROS [2, 10]. Furthermore, since the phosphate group of DNA is negatively charged, DNA is a polyanion in aqueous solutions [11]. Therefore, the phosphate groups' counter ions, especially Na+, play a crucial role in the reaction of the radical cation with water [11]. Irrevocable reaction of radicals with H_2O in DNA helix preferentially happens at the most oxidative sites. In fact, the reaction widely occurs with the guanine, 8-oxo-7,8-dihydroguanine (8-OxodG), and possibly with thymine (thymine glycol) which are known as oxidation products [11]. Consequently, oxidative stress may cause damage to several cellular molecules including lipids, proteins, and nucleic acid resulting in gene expression and finally leads to cell death [12]. Clearly, a single entire comet consists of a head and a tail. The head represents the undamaged DNA that remains in the cell nucleus whereas the damaged DNA fragments usually migrate away from the head but still connect to the head making the tail of the comet become a relaxed ssDNA thread. This damage can be measured by various approaches [13, 14], however the simplest and the easiest way for measuring this damage is the comet assay. Therefore, the aims of the current stud were to detect the DNA damage levels of the human epithelial bladder cancer cell lines (RT112) by using the alkaline comet assay, and to investigate whether or not the measurement parameters are correlated.

2. MATERIALS AND METHODS

2.1. Cell lines

The human epithelial bladder cancer (RT112) cell lines were used in this study. The characterization and derivation of these cell lines have been described previously [15].

2.2. Cell culture

The RT112 cells were cultured in cell-seeded 6-well plates. The cell lines were cultured with 10% fetal bovine serum (FBS), penicillin G (45 $\mu g \cdot ml^{-1}$), streptomycin (45 $\mu g \cdot ml^{-1}$), and kanamycin (90 $\mu g \cdot ml^{-1}$), at 37 °C in a 5% CO_2 humidified incubator. 70 to 80% confluent culture of this cell line were taken to minimize the variability and/or overexpression of H_2O_2 during different phases of cell cycle.

2.3. Cell treatments

The comet assay was performed under alkaline conditions according to the technique described by Singh *et al*. [16]. Briefly, the cell lines underwent alkaline buffer unwinding and electrophoresis for a short run-time. Subsequently, the nucleoid containing strand breaks extends in the direction of the anode and forms the tail of the comet, while intact DNA stays in the head of the comet. Four different dilutions (concentrations) from 10 mM H_2O_2 stock solution and serum-free medium were made. The cells were incubated with 10 ml of H_2O_2 of four different concentrations (0, 10, 20, and 50 µM) for 30 minutes on ice, and covered with aluminium foil to avoid light-induced DNA damage formation. The sections were immediately washed using pre-warmed phosphate buffered saline (PBS). The cells then were incubated with 500 µl of the pre-warmed trypsin/ethylenediaminetetraacetic acid (EDTA) solution for 5-6 minutes at 37 °C. After the cells were detached from the plates, 1 ml of complete medium was added into all the wells. The cells were pelleted in eppendorf tubes by centrifugation at 2000 r.p.m for 4 minutes in a cold room. The cells were suspended in 0.6% low melting point agarose in PBS. Glass microscope slides were precoated by embedding them in 1% normal melting point agarose. This was followed by the addition of 80 µl of the cell/agarose mixture to the centre of the precoated slides which were then covered with coverslips to get a thin layer of agarose. Slides were left on ice for 5-10 minutes to set the gel, and the coverslips were then removed. All the slides were placed into a coplin jar containing lysis buffer (100 mM disodium EDTA, 2.5 M NaCl, 10 mM Tris-HCl, pH 10.0 with sodium hydroxide plus 1% triton X-100) at 4 °C overnight in order to remove all cell debris like proteins.

2.4. Single-cell gel electrophoresis assay

SCGE assay is based on a previous study [16] with minor modifications of the method. The slides were washed twice with chilled double distilled water and with fresh water for 5 and 10 minutes, respectively. The samples were subsequently electrophoresed at 30 V and 300 mA for 20 minutes at 4 °C in an electrophoresis buffer (300 mM NaOH, 1 mM Na_2EDTA, pH >13). After this step, the slides were neutralised by incubating with neutralization buffer (0.4 M Tris-HCl, pH 7.5) for 20 minutes.

The slides were then washed with fresh tap water for 10 min, followed by drying in the oven at 37 °C. After the slides were dried, they were stained with propidium iodide (2.5 μg/ml; ~ 1 ml per slide) for 20 minutes at room temperature in the dark. The slides were rinsed with distilled water for 30 minutes; this was followed by the drying of the sections in the oven again at 37 °C. All aforementioned steps were performed under dimmed light to avoid further DNA damage by light. Finally the comets were visualised by using a fluorescence microscope and a camera connected to it, and analysed by using the Komet analysis software from Andor Technology (Belfast, UK). Fifty cells per slide were randomly picked up for measuring, and a total of 100 cells per each concentration were scored.

2.5. Statistical analysis

Statistical analysis was used to evaluate the changes that happened due to H_2O_2 at different concentration points. GraphPad Prism version 6 (GraphPad software, La Jolla, California, United States) and Microsoft Excel program were used in this experiment. One-way analysis of variance (ANOVA) was carried out, followed by post-hoc analysis (Tukey's test) to compare between multiple concentration points of H_2O_2. Results at p value < 0.05 were considered as statistically significant.

3. RESULTS

Figure 1 shows the percentage of DNA in the tail of comets of RT112 cell line after being treated with four doses of H_2O_2 for 30 min. It can be seen that by increasing the dose of H_2O_2 on the cells, the percentage of DNA in the comet's tail was increased. There are two dose points in the curve, at which tail DNA % was extensively different when compared to that in untreated control cells. At 20 μM H_2O_2, the % of DNA in the tail was slightly higher than that in control cells. However, at a higher concentration of H_2O_2, 50 μM, tail DNA % was almost doubled compared to that in untreated control cells (from ~15% to above 30%). Interestingly, the values of these two concentration doses of H_2O_2 (20 μM and 50 μM) are not only significant when compared to background damage levels, but also significant when compared to their lower concentrations. For instance, the values at 20 μM H_2O_2 are significant when compared to those at 10 μM ($p = 0.03$), and those at 50 μM are also significantly different when compared to those at 20 μM H_2O_2 ($p = 0.0004$) (one-way ANOVA, Tukey's test). The percentage of DNA in the tail was below 50% at all of these H_2O_2 concentrations, and a good linear correlation was noticed between the dose and the percentage of tail DNA.

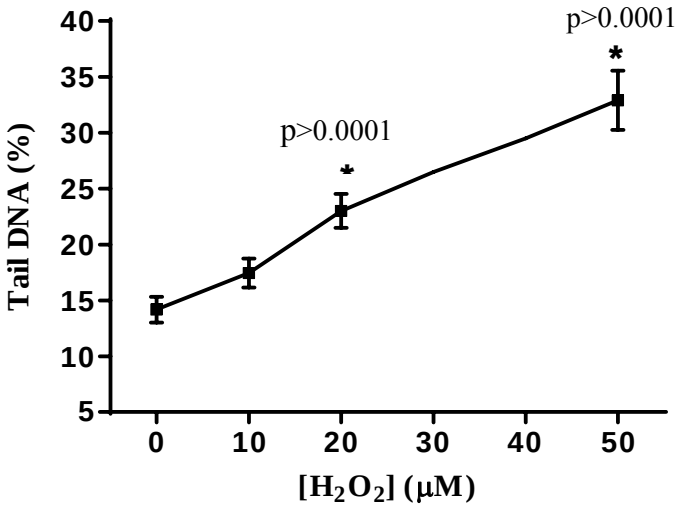


Figure 1. DNA percentage in the tail of comets after being treated with H_2O_2 at four doses for 30 min. One hundred cells per sample were analysed under fluorescence microscope and by using the Komet software, followed by one-way ANOVA Tukey's test. The error bars in the data are represented as the mean ± SEM. Significant at ***** p value < 0.0001 when compared to untreated cells.

Turning to length of the comet tails, it is clear that the tail length of comets was increased gradually by increasing the dose of the damaging agent. At low doses of H_2O_2, concentrations between 0 µM to 20 µM, there was a steady growth in the length of the comet tails (from just above 20 micrometres to roughly 30 µm in length). Moreover, there was a slight stabilization in tail length of comets at a high dose of H_2O_2, 50 µM, although this was considered as significantly different when compared to untreated cells. Additionally, the length at 20 µM H_2O_2 concentration was also considered to be markedly different compared to background levels in untreated control cells ($p = 0.004$) (Figure 2). There is a limitation concerning this method when it comes to the measurement the length of tails in comets as they reach their peak after exposure of the cell lines to higher doses of H_2O_2.

Figure 3 gives the information about the correlation between the % of DNA in comet tails and lengths of tail comets of the cell populations. It is noticeable that the lengths of the comets of the H_2O_2-treated cells were established quickly. For instance, DNA is sensitive enough to be damaged at a low and very initial concentration of H_2O_2 and the tails of comets are formed. Clearly, the analysis of this data revealed that there was a good positive correlation between tail length and tail DNA % of the comets formed in the H_2O_2-treated RT112 cells ($r^2 > 0.98$).

4. DISCUSSION

In the current study, the H_2O_2-induced DNA strand breaks at various levels in the RT112 cell line was detected by the SCG unwinding assay. To obtain clear and fundamental results of DNA damage produced by H_2O_2, several factors should be considered such as the alkaline unwinding treatment time and electrophoresis running time as well as the pH measures of the solution [17]. These conditions, however, might not be same for all cell types. For example, a certain type of cell might need a pH or electrophoresis running time different from those of others to get better results. A previous study concluded that "pH >13, 10-min alkaline unwinding treatment and 20-min electrophoresis run time" gives the most optimal results for a particular human cell lymphocyte [4]. A great diversity of parameters could be detected by the software, including tail length, tail DNA %, tail moment and Olive tail moment [18]. However, the most preferred parameters of DNA migration used to reflect the

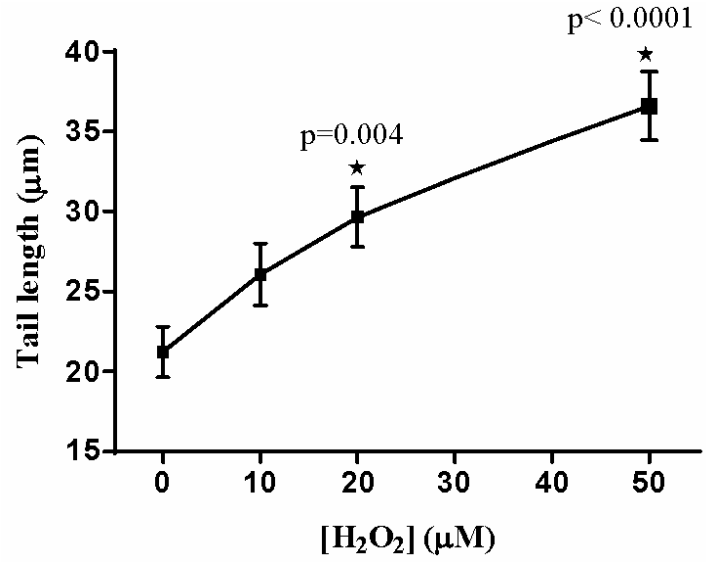
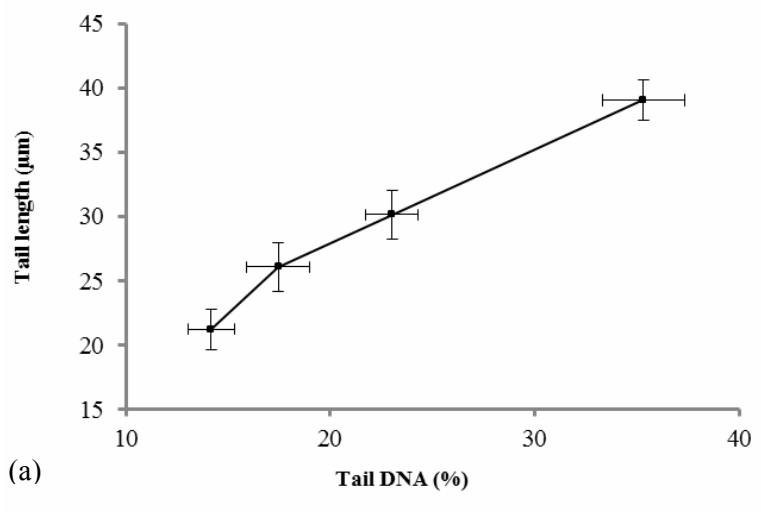


Figure 2. Tail length of the comets after human epithelial bladder cancer (RT112) cell line was incubated with four different doses of H_2O_2 for 30 minutes. Lengths of the comets were measured in micrometres (µm). One hundred cells were scored per each dose; the data is represented as mean ± SEM by one-way ANOVA. Star symbols (★) indicate that the values are significantly different from the control level of damage.



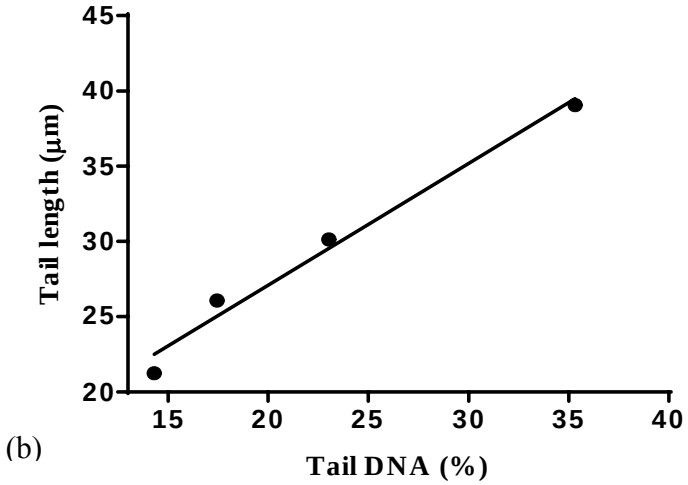


Figure 3. Positive relationship between tail DNA (%) and tail length (µm) of comets of the human epithelial bladder cancer cells after being treated with different doses of H_2O_2 for 30 min. (**a**) Microsoft excel program (mean ± SEM), (**b**) linear regression using Prism 6 software. $p = 0.009$, $r^2 = 0.9812$.

DNA damage are tail length and tail DNA %. In general, by increasing the dose of the damaging agent, the percentage of tail DNA (tail DNA %) and lengths of comet tails were increased dramatically. These results were supported by a previous study [12].

Lengths of tail comets at low doses of H_2O_2 showed constant increase while at high doses were almost levelled off. Therefore, tail length has not been a satisfactory tool for measurement of DNA damage [14]. Tail length is considered to be increased at low damage levels, and it is relatively sensitive to the background damage levels; hence statistical procedure might be affected [13]. In addition, by increasing the dose of H_2O_2, only the intensity of the tails in comets increases but it does not affect the length of the tails because the tails are only as long as the piece of a single broken DNA linked to the head [14]. It means that by raising the dosage level of H_2O_2, the % of DNA in the tail and the intensity of the tail are increased. In other words, increasing the dose of the damaging agent (H_2O_2) will not affect the length of the tail; only the intensity of the tail will be higher (this is considered as percentage of DNA damage).

Each individual comet consists of a head and a tail. The head of the comet contains supercoiled

unbroken intact DNA (dsDNA), while the tail consists of broken relaxed single strand DNA (ssDNA) [19]. When the cells treated with H_2O_2, the amount of DNA in the head decreases whereas the amount of DNA in the tail increases. As a result, if cells were exposed to higher (non-lethal) doses of H_2O_2, virtually all the damaged DNA would assemble in the tail, and this would lead to 'hedgehog' comets [20]. Previous studies were strongly encouraged to report the % of DNA in tails rather than tail length of the comets [13]. Several previous studies have indicated that DNA % in the tail of the comets is the most profitable parameter to be measured [21, 18].

A number of previous studies have highlighted the use of microscopic slides for measuring the DNA damage when there is no other equipment available [21, 18]. However, this method might be unreliable and would not give the exact values as the software does, because the investigation of association between the % of DNA in tails and tail length (μm) may be biased.

It is well known that different image analysis methods give different values during measurement of most comet assay parameters. Therefore, it would be difficult to compare the results in the inter-laboratory computation systems because different software packages use algorithm variations according to the DNA distribution profile in the tail. Under these conditions, it is highly recommended to use percentage of tail DNA for scientific regulatory purposes and for inter-laboratory comparisons [17]. The best way to describe results in comets' application analysis is to normalise the results when comparing the treated cells to control because the untreated (control) cells might give comets with a background damage of about 10% of DNA in the tail [14].

5. CONCLUSION

The alkaline form of the comet assay is potentially a good versatile approach, and is a more effective, highly sensitive and productive way for investigation of DNA damage levels in small populations of single cells. The results of this study revealed that % of DNA in tail comets is a good indicator of DNA damage. The percentage of DNA in the tail of comets illustrates a variety of DNA damage. There was a strong positive correlation between the length of tail comets and the % of DNA present in it.

ACKNOWLEDGMENTS

The author is delighted to express his sincere gratitude to the Department of Cancer Studies and Molecular Medicine at the University of Leicester for providing laboratory facility.

CONFLICT OF INTEREST STATEMENT

The author declares that no competing interests exist.

REFERENCES

1. Shukla, A. K., Pragya, P. and Kar Chowdhuri, D. 2011, Mutation Research, 726, 222-226.
2. Loft, S. and Poulsen, H. E. 1996, J. Mol. Med., 74, 297-312.
3. Kang, S. W., Rhee, S. G., Chang, T., Jeong, W. and Choi, M. H. 2005, Trends in Molecular Medicine, 11, 571-578.
4. Lee, R. F. and Steinert, S. 2003, Mutation Research, 544, 43-64.
5. Liu, Y. and Hu, N. 2009, Biosensors and Bioelectronics, 25, 185-190.
6. Shi, S., Hudson, F. N., Botta, D, McGrath, M. B., White, C. C., Neff-LaFord, H. D., Dabrowski, M. J., Singh, N. P. and Kavanagh, T. J. 2007, Cytometry Part A, 71A, 686-692.
7. Szatrowski, T. P. and Nathan, C. F. 1991, Cancer Res., 51, 794-798.
8. Greaves, M. and Maley, C. C. 2012, Nature, 481, 306-313.
9. Griendling, K. K. 1997, Circulation, 96, 3264-3265.
10. Weyemi, U. and Dupuy, C. 2012, Mutation Research-Reviews in Mutation Research, 751, 77-81.
11. Kanvah, S., Joseph, J., Schuster, G. B., Barnett, R. N., Cleveland, C. L. and Landman, U. 2010, Accounts of Chemical Research, 43, 280-287.
12. Behravan, J., Mosafa, F., Soudmand, N., Taghiabadi, E., Razavi, B. M. and Karimi, Gh. 2011, J. Acupunct. Meridian Stud., 4, 193-197.
13. McKenna, D. J., McKeown, S. R. and McKelvey-Martin, V. J. 2008, Mutagenesis, 23, 183-190.

14. Collins, A. R. 2004, Molecular Biotechnology, 26, 249-261.
15. Fujiyama, C., Jones, A., Fuggle, S., Bicknell, R., Cranston, D. and Harris, A. L. 2001, British Journal of Cancer, 84, 558-564.
16. Singh, N. P., McCoy, M. T., Tice, R. R. and Schneider, E. L. 1988, Exp. Cell Res., 175, 184-191.
17. Kumaravel, T. S. and Awadhesh, N. J. 2006, Mutation Research, 605, 7-16.
18. Garcia, O., Romero, I., González, J. E, Moreno, D. L., Cuétara, E., Rivero, Y., Gutiérrez, A., Pérez, C. L., Alvarez, A., Carnesolta, D. and Guevara, I. 2011, Mutat. Res., 720, 14-21.
19. Petersen, A. B., Gniadecki, R., Vicanova, J., Thorn, T. and Wulf, H. C. 2000, Journal of Photochemistry and Photobiology B: Biology, 59, 123-131.
20. Szeto, Y. T., Lee, A. K. H., Benzie, I. F. F. and Obied, H. K. 2012, Human and Experimental Toxicology, 31, 1144-50.
21. Collins, A. R., Oscoz, A. A, Brunborg, G., Gaivão, I., Giovannelli, L., Kruszewski, M., Smith, C. C. and Stetina, R. 2008, Mutagenesis, 23, 143-151.