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A brief study using comet assay to assess the extent of DNA damage in random individuals

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Abstract
DNA damage is described as any kind of alteration in the structure of the nucleic acid with a possibility of change in the function depending up on the severity of damage. Such alterations impact the viability and cellular machinery. Such damages turn out to be major reason for cancer and other tumours. A mutation originated from any DNA damage progresses further developing into a cancer. These DNA alterations can be induced through a wide range of physical and chemical mutagens. A cluster of mutations that activate cancer causing genes (oncogenes), inactivate suppressor genes and disturb genome stability pave path to cancer. With this reference, we consider the history of smoking in few individuals to briefly assess their DNA using comet assay. Comet assay traditionally described as 'single cell gel electrophoresis' assay is being employed to detect DNA strand breaks. The formation of 'comets' can be traced as there is variation in the migration of damaged DNA and non-damaged DNA. The assay comes up with advantages such as better sensitivity, quick application, and flexibility to perform but on contrary, it cannot detect mitochondrial DNA damage.

Keywords: comet, transversions, carcinogen, fixation and lymphocytes

Introduction
Often in the lab, clinical trials and studies are performed to assess DNA damage. One of the carcinogenic activities affecting the DNA of an individual is smoking. Smoking is associated with lung cancer. Smokers carry a high mutational load. Previous studies have emphasised on p53 mutations that are very frequent cancers coupled with tobacco exposure. The mutational pattern observed is 'transversions' i.e G to T transversions which are distinct in smoking individuals. The occurrence of these transversions is 30% in lung cancer associated with smoking individuals whereas 12% in lung cancer of non-smoking individuals. Tobacco related cancers have demonstrated G to T transversions in most cases but non-tobacco related cancers seem to be more of G to A transitions. The 2 major carcinogens in concern are Polycyclic Aromatic Hydrocarbons (PAH) and nicotine derived nitrosamines. A connection exists between the formation sites of PAH adducts and transversion hotspots in lung cancers. Other carcinogens too contribute to development of cancer via the G to T transversion mutations. An instant damage to the DNA from cigarette smoke is a consequence of the p53 mutations. The extent of DNA damage caused through such mutagens can be assessed through Comet Assay to determine the extent of carcinogenic activity in an individual's DNA.

Materials and methods
(It is to be noted that the physical measurements of the materials and the procedure employed can be modified according to convenience of the study and laboratory, provided it does not alter the composition entirely).

Reagents
1. Phosphate Buffered Saline (PBS)
- 4g NaCl
- 100 mg KCl
- 5.75g Na_2HPO_4
- 200 mg- KH_2PO_4
- Dissolve in 1000ml distilled water
- pH – 7.2

2. Lysing solution
For 100 ml
- 14.6g- NaCl
- 3.7g- EDTA
- 0.12g- Tris
- pH- 10.5

For 500ml
- 82.07g-NaCl
- 20.89g-EDTA
- 0.67g-Tris
- Ph-10.5

- The above components are dissolved in 700ml distilled water and continuously stirred for 15 minutes using a magnetic stirrer. 10g of Sodium Lauryl Sulphate (SLS) which is an anionic detergent is added and stirred again. pH is adjusted to 10 with concentrated HCl or NaOH and final volume made up to 890ml and kept at room temperature.
- Before adding it to the slide, 1% Triton-X and 10% DMSO was added to the above lysing solution and chilled for 20-30 minutes.

3. Electrophoresis buffer (TAE)
For 500ml distilled water
- 15ml NaOH
- 2.5ml EDTA

While working, it is preferable to make buffer solution fresh before each run. 30ml of NaOH solution was mixed with 5ml EDTA solution and volume is elevated to 1000ml using distilled water.

4. Neutralization Buffer

- 0.4M Tris (48g) is added in 1000ml distilled water and pH is set to 7.5 with conc. HCl.

5. Fixing solution (100ml)

- TCA-15g
- Zinc sulphate- 5g
- Glycerol- 5g

6. Silver Staining Solution

Staining Solution A-

- 5g Sodium carbonate in 100ml water

Staining Solution B- **(In 100ml of water)**

- Ammonium nitrate- 190mg
- Silver nitrate- 200mg
- Tungstosilicic acid- 1000mg

Before utilizing these solutions, add 32ml A and 16ml B which represents working solution C, this mixture is poured on to dried gel slides.'

The stopping solution here is 1% Glacial Acetic Acid, once staining is finished, the slides are dipped in the stopping solution.

Method

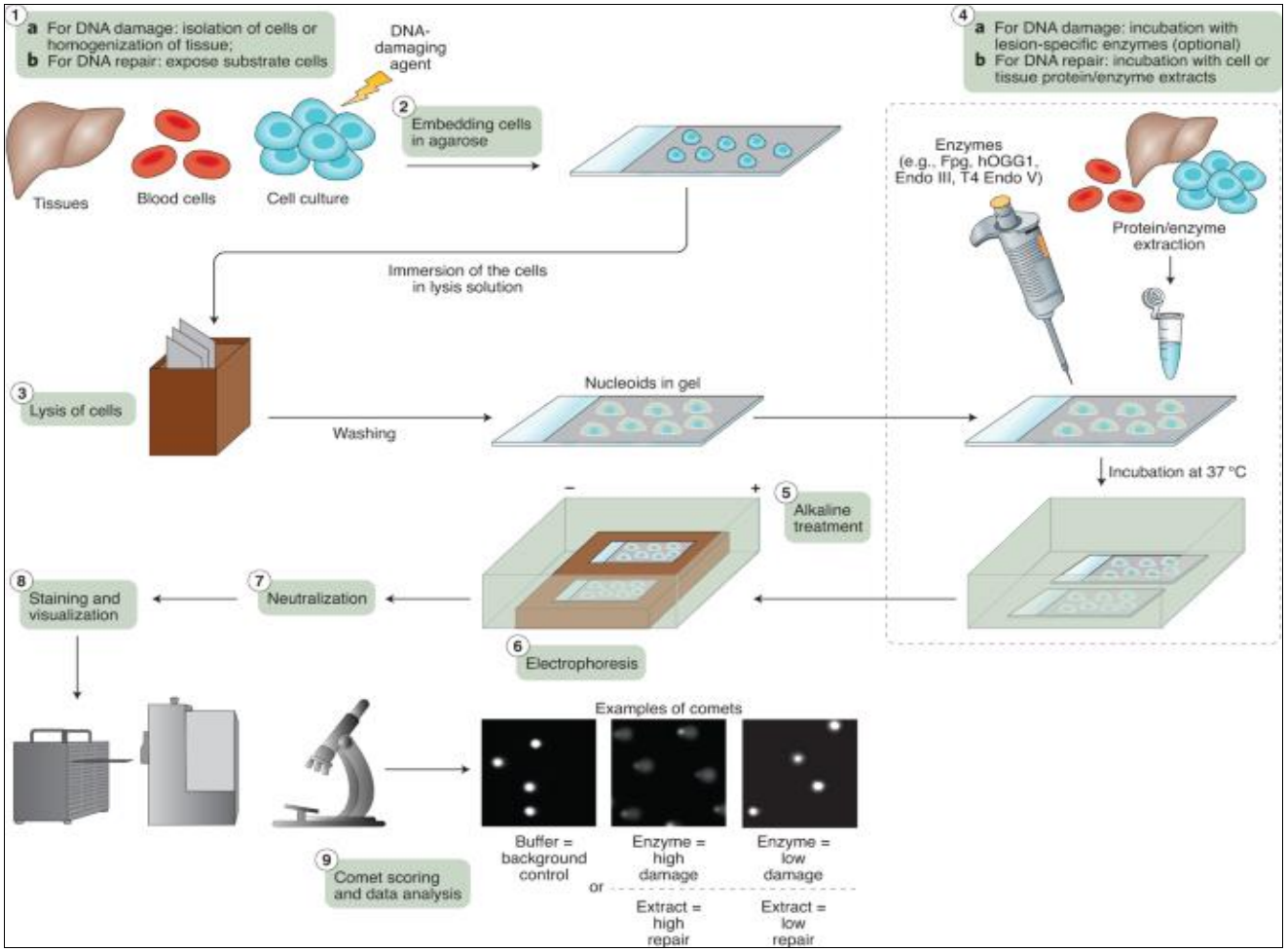

Fig 1

Step 1: Preparation of Pre-Coated Slides

- Sterile and plain glass slides are dipped in hot 1% NMPA (Normal Melting Point Agarose) and one of the sides is wiped off.
- Slides are dried overnight at 37°C.

Step 2: Layering of gels

- Pre-coated slides were layered with a mixture of 1% LMPA (Low Melting Point Agarose) and 20 µl of blood.
- Under sterile conditions only, the blood must be collected and stored till use.
- A cover slip is placed and allowed to set for 10 minutes.
- The cover slip is slide off later.
- It is performed under dim light.

Step 3: Layering of cells

- The slides were layered with 110µl of LMPA for the 3rd agarose layer.
- A cover slip was placed on the gel and allowed to set for 10 minutes.
- The cover slip is slide off later.

Step 4: Lysing of cells

- The lysing solution prepared is now employed to lyse the cells.
- Slides are placed in the lysing solution for 4hrs at 4°C.
- It is performed under dim light.

Step 5: Unwinding
- Slides are taken out from the lysing solution and placed side by side in the horizontal electrophoresis box.
- The chamber is filled with alkaline buffer in such a way that the levels of buffer completely cover the slides.
- Care is to be taken to avoid bubble formation.
- Slides are immersed in the buffer for 30-40 minutes for the DNA to unwind.

Step 6: Electrophoresis
- Voltage is maintained at 25 V and current at 300mA.
- The run is carried out for 30 minutes.
- Afterwards, slides are transferred to a staining tray.

Step 7: Neutralization
- 0.4M Tris neutralizing buffer is used to neutralize the slides.
- After 10 minutes of settlement, the buffer solution is drained off.

Step 8: Fixation of Slides
- Slides are exposed to fixing solution for 10 minutes and there after washed with distilled water.
- Slides are air dried at 37° C for couple of hours or overnight.

Step 9: Staining
- It is to be performed only in dark.
- Staining solution C prepared is spread uniformly over the slides.
- Slides are gently shaken for 20 minutes and it is repeated for a couple of times using the same solution till a faint grey colour appears.
- Lastly, the slides are run down 2 times using distilled water.

Step 10: Stopping
- A jar is filled with stopping solution and the slides are immersed gently into it for 5 minutes till a yellowish-brown colour appears.
- Slides are washed with distilled water and placed at an inclined position under normal room temperature.
- This procedure is done in dark.
- Now the slides are employed for observation under the microscope.

Fig 2

Results

The protocol has been followed using blood samples of 2 individuals making it clear that the work carried out was on a very small scale. The 2 individuals have a vague history of smoking i.e exposure to carcinogens (though only to a little extent), allowing their DNA to be analysed for appearance of comets under the microscope. The study did not have a formal conclusion since we had limited the work up to only 2 individuals. Previous laboratory studies had employed comet assay for assessing level of DNA damage in lymphocytes of patients with recurrent pregnancy loss compared to the controls.

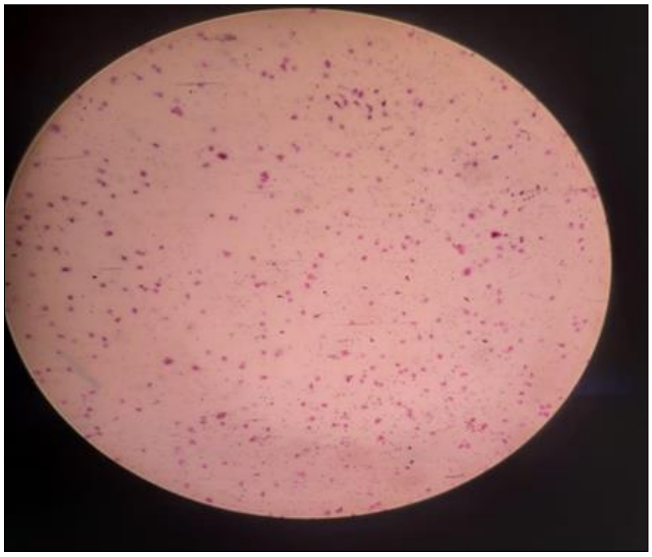

Fig 3: Sample A

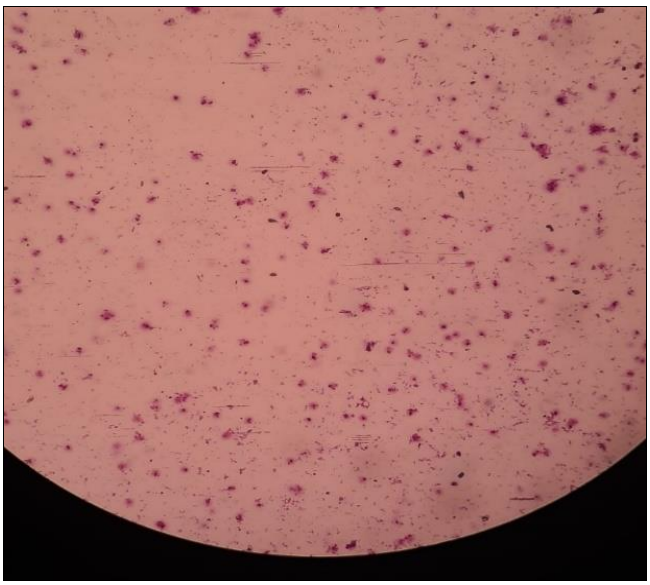

Fig 4: Sample B

Under the microscope, the results observed illustrate the extent of DNA damage which seems to be very negligible owing to the absence of formation of many comets. As DNA damage increases, the tail length of the comet is expected to gradually increase and reach a maximum point, this relies on the electrophoretic conditions.

Conclusion

Due to absence of comet formation, it can be noted that there has been no distinct DNA damage caused due DNA cross linkages, oxidative base damage, alkali labile sites, etc. This laboratory work is very brief and conducted only to interpret the results of the assay and not to draw extreme conclusions. The volume of samples could be elevated for a wider study and better comparison besides modifying the protocol to suffice the requirements of the study.

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