The use of different blood cell preparations in the comet assay

Siv Bøhn, Vaineta Vebraine, Sergey Shaposhnikov
and Andrew Collins

NorGenotech AS and University of Oslo

hCOMET CA15132
Traditionally, the material most often used for the comet assay in human biomonitoring is blood, or specifically peripheral blood mononuclear cells (PBMCs). They are commonly referred to as lymphocytes, which are the major component of PBMCs, though there are also some monocytes.

PBMCs can be analysed immediately, or after storage at -80° or below. Slow freezing is recommended, and the freezing medium normally contains dimethylsulphoxide (DMSO) to prevent ice crystal formation. Thawing is also critical, as cell membranes are damaged by exposure to DMSO at temperatures above freezing.

At first, human PBMCs were simply analysed for DNA breaks, as an indicator of, for example, exposure to DNA-damaging chemicals or radiation, or disease. However, variants of the comet assay were soon developed for other kinds of measurement:
The application of the comet assay to monitor DNA repair was described in the earliest comet assay papers; lymphocytes were X-irradiated and then incubated, and the decrease in DNA breaks was followed with time. Since then, more sophisticated methods have been developed.

In early trials of antioxidant nutrients, lymphocytes were treated ex vivo with H$_2$O$_2$, which induces DNA breaks. A decrease in these breaks in supplemented subjects (compared with controls) is regarded as an indication of the effectiveness of the antioxidant.

Lesions other than strand breaks are often more informative. For example, including in the comet assay a digestion of DNA with endonuclease III or formamidopyrimidine DNA glycosylase [Fpg] (bacterial repair enzymes converting oxidised pyrimidines and purines respectively into DNA breaks) allows the quantitative assessment of DNA damage resulting from oxidative stress.
The comet assay (modified to detect damaged bases)

- Cells embedded in agarose on microscope slide
- Lysis: Triton X-100, 2.5 M NaCl
- Nucleoids; supercoiled DNA
- Alkaline incubation: 0.3 M NaOH, 10 mM EDTA
- Electrophoresis: 0.8 V/cm, 30 min
- Neutralisation, DAPI stain, fluorescence microscopy
- ± Digestion with lesion-specific endonuclease

The frequency of damaged bases is given by the increase in DNA breaks in the presence of the specific endonuclease.

This assay allows us to estimate the level of DNA base damage in (e.g.) white blood cells.
PMNCs have disadvantages. It takes time to isolate them (several centrifugations are needed), and when numerous blood samples are to be analysed on one occasion – as in most human biomonitoring trials – this is a serious constraint. It may mean that samples are left for varying times on the bench or in the fridge until enough have accumulated to carry out the isolation together.

So what are the alternatives?

It was shown a few years ago, by Al-Salmani et al., that whole blood frozen in small volumes – with or without DMSO, and without the need for slow freezing – could subsequently be used in the comet assay to measure strand breaks.

Leukocytes from frozen blood

Whole blood cannot be used in the version of the assay involving induction of strand breaks with \( \text{H}_2\text{O}_2 \): the \( \text{H}_2\text{O}_2 \) is simply broken down (by catalase from the red cells, or haem catalysing a Fenton reaction) before it can exert its effect on DNA. So we decided to try isolating leukocytes from frozen whole blood.

**Procedure:**
- Freeze blood in small aliquots (~250 µL) on dry ice or in liquid N2.
- Thaw blood, but don’t allow to warm; immediately add 100 µL to 1 ml of cold PBS. Freezing and thawing lyses red cells, so the leukocytes are suspended in a red PBS solution.
- Centrifuge, 200xg, 5 min, 4°C.
- Suspend pellet in 1 ml of cold PBS; cell count on sample; centrifuge again.
- Repeat twice. The purpose of washing is to get rid of traces of red cells, especially catalase and haem which break down \( \text{H}_2\text{O}_2 \).
- Embed cells in agarose and carry out comet assay as usual.
Leukocytes were isolated from whole blood samples from 10 subjects, frozen for \(~1\) year.

Strand breaks (SBs), Fpg-sites, and \(\text{H}_2\text{O}_2\)-induced breaks were measured – in whole blood...

...and in isolated leukocytes

H$_2$O$_2$-induced breaks and endogenous Fpg-sensitive sites (oxidised purines) in the DNA both reflect individual antioxidant status.

There was a good correlation between H$_2$O$_2$-induced breaks and Fpg-sensitive sites.

This agreement suggests that what we are measuring has real biological significance.
An important question is whether similar levels of damage are present in the whole white cell population and in the sub-fraction of PBMCs. We compared frozen whole blood and isolated PBMCs, from a group of colorectal cancer patients.

Regarding strand breaks, the two cell populations show a good correlation – though mean values at the individual level differ (more damage in whole blood).

There was significantly more damage (strand breaks) in the (whole blood) DNA of patients receiving chemotherapy compared with those not – even after adjusting for stage of disease, and age and sex.

Kværner et al. (2018). DNA Repair 63, 16-24
Whole blood → PBMCs

frozen, thawed

leukocytes

SBs, Fpg, H2O2

SBs, Fpg

SBs, Fpg, H2O2
Leukocytes from frozen buffy coats?

When fresh blood is centrifuged, the top layer of the red cell fraction is enriched in leukocytes. This layer – known as the **buffy coat** – is often stored frozen in straws in biobanks. It represents another possible source of material for biomonitoring. We isolate **leukocytes** similarly as from whole blood.

**Comet assay compatible?**

```
Buffy coat  →  frozen, thawed  ←  leukocytes
```

- SBs, Fpg
- SBs, Fpg, H₂O₂
White cells isolated from 12 frozen buffy coat samples (healthy subjects)

- Low levels of SBs
- Variation in levels of Fpg sites and H$_2$O$_2$-induced breaks
- Significant correlation between Fpg sites and H$_2$O$_2$-induced breaks

[Unpublished data, Vaineta Vebraite]
How do buffy coat leukocytes compare with PBMCs?

In a new set of samples, we compared mean damage levels in buffy coat, isolated buffy coat leukocytes, and PBMCs.

More SBs and Fpg-sites in buffy coat leukocytes. H$_2$O$_2$-induced breaks at similar levels in leukocytes and PBMCs.

[S.K. Bøhn et al., submitted]
In spite of the higher level of strand breaks in leukocytes, there is a good correlation (but less good concordance) between leukocytes and PBMCs.
Fpg sites show high correlation and good concordance between leukocytes and PBMCs.
The ability to store whole blood or buffy coat frozen before isolating leukocytes has advantages over the isolation of PBMCs from fresh blood – a time-consuming process. At least qualitatively, leukocytes and PBMCs give comparable data on SBs, oxidised bases (Fpg-sites) and $\text{H}_2\text{O}_2$-induced breaks.

Although white blood cells are not cancer target cells, they provide useful and relevant information on DNA damage. Ø They circulate through the body, and so reflect whole body exposure. Ø They are easily obtained. Ø There is some evidence that DNA damage in PBMCs correlates with damage in normal colon tissue, for example.

*If cells can be isolated from buffy coat after long-term storage, we should be able to measure DNA damage in cohort studies.*