Detection of DNA damage after hyperbaric oxygen (HBO) therapy

Claudia Dennog¹, Andreas Hartmann¹, Günter Frey² and Günter Speit¹,³

¹Universität Ulm, Abteilung Medizinische Genetik D-89069 Ulm, and
²Bundeswehrkrankenhaus Ulm, Abteilung Anästhesie u. Intensivmedizin, Germany
³To whom correspondence should be addressed

Hyperbaric oxygen (HBO) therapy is successfully used for the treatment of a variety of conditions. However, exposure to high concentrations of oxygen is known to induce damage to cells, possibly due to an increased oxygen radical production. As reactive oxygen species also cause DNA damage, we investigated the DNA-damaging effect of HBO with the alkaline version of the single cell gel test (comet assay). Oxidative DNA base modifications were determined by converting oxidized DNA bases to strand breaks using bacterial formamidopyrimidine-DNA glycosylase (FPG), a DNA repair enzyme, which specifically nicks DNA at sites of 8-oxo-guanines and formamidopyrimidines. HBO treatment under therapeutic conditions clearly and reproducibly induced DNA damage in leukocytes of all test subjects investigated. Increased DNA damage was found immediately at the end of the treatment, while 24 h later, no effect was found. Using FPG protein we detected significant oxidative base damage after HBO treatment. DNA damage was detected only after the first treatment and not after further treatments under the same conditions, indicating an increase in antioxidant defences. DNA damage did not occur when the HBO treatment was started with a reduced treatment time which was then increased stepwise.

Introduction

Hyperbaric oxygen (HBO) therapy implies the inhalation of 100% oxygen under pressures not lower than 2 ATA (atmospheres absolute) and for times not shorter than 60 min as delineated by the European Consensus Conference on Hyperbaric Medicine in 1994 in Lille (France). Various therapeutic uses for HBO are well established and controlled HBO has been used successfully for the treatment of a variety of conditions. Commonly accepted clinical indications for its use include decompression illness, acute carbon monoxide (CO) intoxication, air embolism, soft tissue infections and ischaemia (Grim et al., 1990; Tibbles and Edelsberg, 1996). On the other hand, it is known that exposure to high concentrations of oxygen causes damaging effects in humans and experimental animals. It has been postulated that the toxic effects of excessive exposure to oxygen are due to an increased oxygen radical production or other reactive metabolites derived from oxygen (Jamieson et al., 1986). Various antioxidant defences exist to protect cells against reactive oxygen species which include enzymes and low molecular mass radical scavengers (Gutteridge, 1994; Sies, 1991; Anderson, 1996). Because antioxidant defences are not completely efficient, increased free radical formation in the body is likely to increase damage.

The term ‘oxidative stress’ is commonly used to refer to this effect (Halliwell and Aruoma, 1991). Several oxygen-derived species can attack DNA, producing distinctive patterns of DNA alterations (Halliwell and Aruoma, 1991; Epe, 1995). DNA damage induced by reactive oxygen species seems to play an important role in the induction of mutations, cancer and various other disease states in man (Halliwell, 1994).

DNA damage can be detected with various genotoxicity tests. The single cell gel test (comet assay) is a well-established genotoxicity test which has been used to detect a broad spectrum of DNA damage with high sensitivity (Fairbairn et al., 1995; Tice, 1995). In this microgel electrophoresis technique, a small number of cells suspended in a thin agarose gel on a microscope slide is lysed, electrophoresed and stained with a fluorescent DNA-binding dye. Cells with increased DNA damage display increased migration of chromosomal DNA from the nucleus towards the anode, which resembles the shape of a comet. In its alkaline version, DNA strand breaks and alkali-labile sites become apparent, and the amount of DNA migration indicates the amount of DNA breakage in the cell (Singh et al., 1988). The comet assay has been successfully used in screening human blood samples for the effects of radiation and chemical mutagens/carcinogens on DNA (Hartmann et al., 1995a; Tice, 1995). It has been suggested that the comet assay is particularly suited for measuring genetic damage caused by oxidative events in humans (Anderson, 1996; Collins et al., 1995; Hartmann et al., 1995b). In this study we applied the comet assay to the investigation of DNA effects in the blood of persons undergoing a regime of HBO exposure, as used therapeutically.

Materials and methods

Test subjects and HBO treatment

Ten healthy male volunteers (non-smokers, aged 25–34 years) gave informed consent to participate in this study. They were exposed to 100% oxygen at a pressure of 2.5 ATA in a hyperbaric chamber for a total of 3 X 20 min periods, interspersed with 5 min periods of air breathing, this being the usual HBO treatment protocol at this hospital (Bundeswehrkrankenhaus, Ulm, Germany). Venous blood samples were taken before HBO exposure, immediately on exit from the chamber and 24 h later. The blood samples were kept on ice and brought to the laboratory where the comet assay was started within 1 h.

Comet assay

Heparinized whole blood (5 μl) was mixed with 90 μl low melting agarose (LMA; 0.5%) and added to fully frosted slides which had been covered with a bottom layer of 300 μl of 0.75% normal melting agarose. The slides were covered with a coverslip and kept in a refrigerator for 5 min to solidify the LMA. The coverslips were removed, a top layer of 90 μl LMA was added, and the slides were again kept cold for 5 min. After removal of the coverslips, the slides were processed as described previously (Speit and Hartmann, 1995). The time of alkali denaturation was 40 min and time of electrophoresis (0.86 V/cm) was 20 min. The presence of oxidative DNA base damage was determined with a modified protocol (Collins et al., 1993) using the bacterial formamidopyrimidine-DNA glycosylase (FPG protein). After lysis, slides were washed three times in enzyme buffer (50 mM NaPO₄; 10 mM EDTA; 190 mM NaCl; pH 7.5), drained and the agarose covered with 200 μl of either buffer or FPG protein (1 μg/ml) in buffer, sealed with a coverslip and incubated for 30 min at 37°C. The time of alkali denaturation was 20 min and all other steps were as described above.
Images of 50 randomly selected cells (25 cells of each of two replicate slides) were analysed from each sample. Measurements were made by image analysis (Comet Assay 5.0; Perceptive Instruments, Haverhill, Suffolk, UK) determining the median tail moment (percentage of DNA in the tail/tail length) of the 50 cells. Differences between mean values were tested for significance ($P < 0.01$) using Student's $t$-test.

Fig. 1. DNA migration (tail moment) in the comet assay with leukocytes of human subjects before, after, and 24 h after a single hyperbaric oxygen (HBO) treatment. Median tail moment of 50 cells per data point.

- $\square$ = before HBO
- $\blacklozenge$ = after HBO
- $\blacksquare$ = 24 h after HBO

Results

Figure 1 summarizes the effects of HBO treatment (3×20 min) on DNA migration in the comet assay with leukocytes from 10 test subjects. Before HBO treatment, DNA migration (tail moment) in all subjects was in the normal range of controls for the test conditions used. The differences between the subjects reflect normal individual variations, as has been described earlier (Hartmann et al., 1994; Betti et al., 1995). Immediately after HBO exposure, a clear increase in DNA migration was found for all subjects. Three of the subjects were tested repeatedly and the same effect was seen in all trials (data not shown). No increase in DNA migration was observed 24 h after HBO. In a few tests, DNA migration was determined 6 h after HBO and there was no increase as well (data not shown). Figure 1 indicates even lower values for the tail moment 24 h after treatment compared with the values before HBO treatment, an observation that was constantly made during the whole study. Figure 2 illustrates the distribution of DNA damage among the blood cells before and after HBO treatment for the 10 subjects shown in Figure 1. It can be seen that after HBO exposure, the majority of cells exhibit increased DNA migration in comparison with the control. Figure 2 also indicates that a normal size distribution for the tail moment was not observed and, consequently, the median was used to compare individual effects (Fairbairn et al., 1995; Betti et al., 1995). The modified comet assay (i.e. additional treatment with FPG protein) was performed with blood samples of seven

Fig. 2. Distribution of DNA damage among leukocytes of human subjects before and after a single hyperbaric oxygen (HBO) treatment 50 cells per sample.
DNA damage detection after HBO therapy

Fig. 3. The effects of FPG protein on DNA migration (tail moment) in leukocytes of human subjects before, after, and 24 h after a single hyperbaric oxygen (HBO) treatment. Mean and SD of seven subjects. □ = standard protocol of the comet assay; ■ = modified protocol of the comet assay with additional formamidopyrimidine-DNA glycosylase (FPG) treatment.

subjects and the mean values of these tests are given in Figure 3. Using the FPG protein to convert oxidative DNA base damage into strand breaks resulted in a small but significant increase in DNA migration of the control values before HBO exposure. This effect might be due to the presence of spontaneous oxidative DNA base damage in human blood, as previously shown for damage detected by endonuclease III (Collins et al., 1993). Immediately after HBO treatment, the increase in DNA migration is strongly enhanced by addition of FPG protein. Furthermore, 24 h after HBO treatment, when the DNA damaging effect cannot be seen any more, there is also no enhancing effect of the FPG protein. Experiments performed with endonuclease III (specific for oxidized pyrimidines) instead of FPG protein resulted in comparable effects, indicating a broad spectrum of induced oxidative damage (data not shown). To test whether an accumulation of DNA damage occurs after repeated HBO treatments, we investigated four subjects undergoing daily HBO treatment over a period of 5 days (according to the standard protocol). Comet assays were performed on the first, the third and the fifth day of the HBO therapy. The results presented in Figure 4 clearly show that there is no accumulation of DNA damage but a clear indication of an adaptive effect. While after the first HBO treatment the comet assay reveals a clear genotoxic effect, no such effect is seen after the third and the fifth day. DNA migration at day 3 and 5 is in the range of the pretreatment control or even smaller. A further time protocol was used to consider the typical weekend pause (2 days) under standard treatment conditions. These experiments demonstrate that after the genotoxic effect on day 1, no induction of DNA damage occurred on day 4 after a 2 day pause (data not shown). This finding suggests that an increased antioxidant protection lasts for more than 2 days. To see whether the generally applied high partial pressure of oxygen can be used in HBO therapy without the observed genotoxic side effect, we changed the standard protocol (i.e. starting with 3×20 min) by increasing the duration of HBO stepwise from 1×20 min to 2×20 min and 3×20 min during the first 3 days (Figure 5). The results clearly show that there is no significant increase in DNA migration at any time point following this treatment protocol.

All values obtained for the four subjects studied were in the range of controls (i.e. median tail moment <0.1).

Discussion

With this study we demonstrate for the first time the induction of oxidative DNA damage by HBO. A clear and reproducible genotoxic effect was seen with the comet assay immediately after HBO exposure (3×20 min at 2.5 ATA), as used therapeutically. This effect was found in all subjects tested and was due to increased DNA damage in the majority of leukocytes. The comet assay is a well established genotoxicity test that detects DNA damage on the single cell level with high sensitivity (Fairbairn et al., 1995; Tice, 1995). In its alkaline version as used in the present study, DNA strand breaks and alkali-labile sites lead to increased DNA migration and provides a measure of DNA damage (Singh et al., 1988). DNA strand breaks and alkali-labile sites represent an important fraction of DNA lesions induced by oxygen-derived species.

Fig. 4. The effects of daily hyperbaric oxygen (HBO) treatments on DNA migration in the comet assay with leukocytes of human subjects. Blood samples were analysed before and after the first, third, and fifth day of HBO treatment. Mean and SD of four subjects.

Fig. 5. DNA migration in the comet assay with leukocytes of human subjects after increasing the hyperbaric oxygen (HBO) treatment time from 20 min on the first day to 40 min on the second day and to 60 min on the third day. Blood samples were analysed before and after the treatment. Mean and SD of six subjects.

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(‘oxidative stress’) (Anderson, 1996). Exposure to oxygen at high partial pressure is known to be damaging to mammalian cells and it has been suggested that the detrimental effects of exposure to high concentrations of oxygen are due to oxidative stress (Jamieson et al., 1986). Recently, an increase in free radicals in the blood from persons undergoing HBO exposure was directly demonstrated by low temperature electron spin resonance (ESR) spectroscopy (Narkowicz et al., 1993). Using the comet assay in conjunction with an enzyme (FPG protein) specific for oxidized DNA bases, we can now provide evidence for the induction of oxidative DNA base damage by HBO. The FPG protein removes 8-oxoguanine, the imidazole ring-opened purines 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua) and 4,6-diamino-5-formamidopyrimidine (FapyAde) as well as small amounts of 8-oxoadenine (Boiteux et al., 1992). The resulting abasic sites are converted into DNA single-strand breaks by the associated endonuclease activity and can be quantified by the comet assay (Collins et al., 1993). Among the DNA base modifications, 8-oxoguanine exerts the highest affinity for the FPQ protein and is proposed to be the biologically most relevant substrate for this DNA repair enzyme (Tchou et al., 1991). Thus, besides the use of high purification liquid chromatography (HPLC) and gas chromatography/mass spectrometry (GC/MS) techniques (Halliwell and Dizdaroglu, 1992) the application of a specific repair enzyme in combination with a sensitive assay to detect DNA strand breaks (e.g. the comet assay) is an excellent approach for the measurement of oxidative DNA damage (Collins et al., 1993; Collins et al., 1995). 8-oxoguanine is one of the major DNA modifications induced by reactive oxygen species and has attracted special attention because it is a mutagenic lesion and induces G to T transversions due to its mispairing properties (Grollman and Moriya, 1993). Oxidative DNA damage has also been implicated in the production of chromosome aberrations (Gille et al., 1993) and induction of chromosome aberrations by HBO therapy has been reported earlier (Guskov et al., 1990). Furthermore, it has been suggested that 8-oxoguanine is directly involved in the process of carcinogenesis (Floyd, 1990). However, a review of the present literature did not support a cancer-causing or growth enhancing effect by HBO (Feldmeier et al., 1994). As well as an involvement in cancer, damage induced by oxygen-derived species also seems to be important for other kinds of disease such as neurodegenerative disease, chronic inflammatory disease and cardiovascular disease (Halliwell, 1994). Therefore, the observed genotoxic effect of HBO treatment should be taken seriously. Although our results indicate rapid repair of the induced DNA damage and an adaptive reaction of the human body, the initial DNA damage of an HBO therapy should be avoided. Our study indicates the first directions for an improved treatment protocol. A simple and effective way to avoid a genotoxic effect is a stepwise increased treatment time which obviously enables the human body to intensify the antioxidant defences. An increase in antioxidant defences after oxidative stress has already been suggested (Anderson, 1996), and the possible mechanisms are under investigation. For example in rats exposed to HBO treatment, an increase in the content of reduced glutathione (GSH) in lung tissue was observed and interpreted as an adaptive process to protect the lung from oxidative stress (Purucker and Lutz, 1992). Taken together, our study provides evidence for a genotoxic effect of HBO therapy due to oxidative DNA damage. Although the biological significance of this effect still needs further clarification, HBO-induced DNA damage should be avoided by modifying the treatment protocol.

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