# Review article

## Genotoxicity of hyperbaric oxygen and its prevention: what hyperbaric physicians should know

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**Key words**
Toxicity, hyperbaric oxygen, oxygen, stress, diving, review article

**Abstract**

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Hyperbaric oxygen (HBO) therapy is used for the treatment of a variety of diseases, but also leads to oxidative stress as a result of increased formation of reactive oxygen species. The consequences may be damage to the lung, the central nervous system and the genome. The oxidative attack on DNA causes, among other damage, single and double strand breaks. Using the comet assay, a well-established genotoxicity test, it was possible to show that a single HBO exposure leads to increased levels of DNA strand breaks in a close dose-effect relationship. On the other hand, it was possible to demonstrate that these strand breaks are repaired rapidly and that, in repeated HBO exposures, DNA strand breaks occur only after the first treatment, not subsequent ones, indicating an induction of protective mechanisms. In healthy organisms, DNA repair and antioxidant mechanisms maintain a steady-state level of damage with minimal risk to the cell or the whole organism, but it cannot be excluded that HBO might lead to a significant mutational burden in situations where antioxidant defence is deficient or overwhelmed. The administration of antioxidants draws an ambivalent picture; Vitamin C, E or even N-acetylcysteine seems to be ineffective to prevent HBO-induced genotoxicity, whereas the orally effective vegetal superoxide dismutase (SOD, Glisodin®) is effective, and, thus, may play a role in the prevention of oxidative DNA damage.

### Introduction

Hyperbaric oxygen (HBO) therapy comprises inhalation of 100% oxygen at supra-atmospheric ambient pressure. HBO has been successfully used for the treatment of a variety of diseases such as decompression illness, acute carbon monoxide intoxication, gas embolism, soft tissue infections, radiation necrosis and impaired wound healing (e.g., in the context of 'diabetic feet'). However, besides its beneficial effects, HBO may also have deleterious effects, not only on the central nervous system (Paul Bert effect) and on the lung (Lorrain-Smith effect)[1], but also on the genome. It is well known that prolonged exposure to normobaric hyperoxia induces DNA damage.[2] Hence, the induction of DNA damage during HBO is a matter of interest, in particular to the consequently raised question whether HBO has a cancer-promoting effect.[3,4]

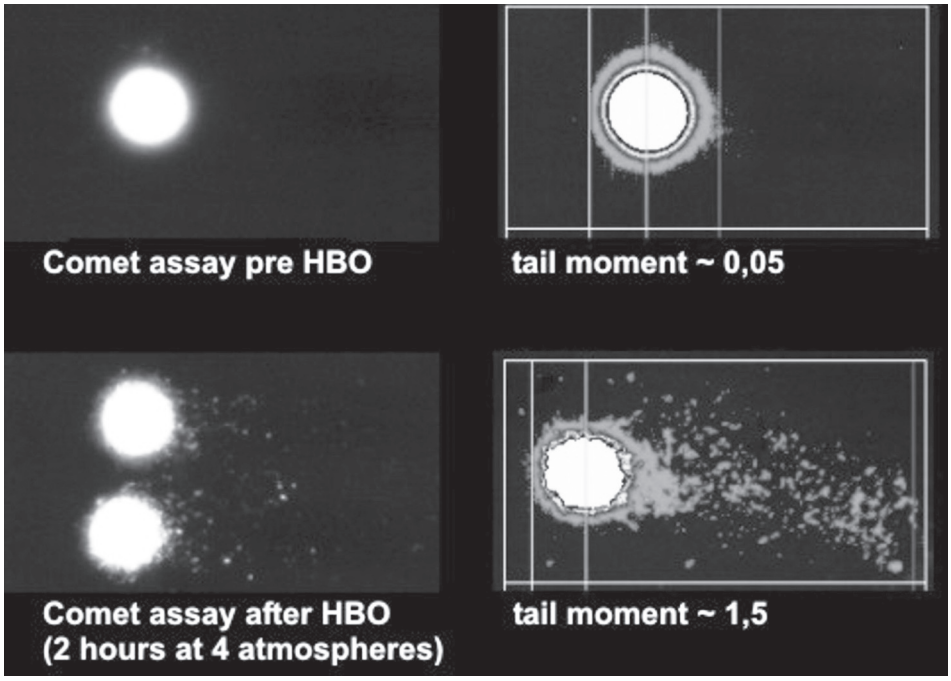
The harmful effects of high oxygen concentrations are due to the abundance of oxygen free radicals, which possess one ore more unpaired electrons.[1,5,6] The collective term 'reactive oxygen species' (ROS) comprises free radicals like $O_2^{\circ}$ and $HO^{\circ}$ as well as non-radical oxygen derivates like hydrogen peroxide ($H_2O_2$) and peroxynitrite ($ONOO^-$).[5,6] ROS are unstable and react with all kinds of cellular compounds, which may result in lipid, protein and DNA damage.[1,5–8] This effect is particularly pronounced in situations of reduced antioxidant defences. Conditions in which ROS production is higher than elimination are called 'oxidative stress', no matter whether they originate from increased ROS formation or decreased elimination.[1,9,10]

Among other cellular structures, the genome is particularly vulnerable, and the possible results of the oxidative attack on DNA are single- and double-strand breaks, abasic sites, 'alkali-labile' sites and oxidized bases.[8,10,11] This, in turn, can lead to mutations, if the lesions are not adequately repaired. The ultimate consequence can be the initiation or the progression of cancer, if specific genes like tumour suppressor genes or oncogenes are affected.[3,12,13] Therefore, there has been worry about the cancer-causing effect of HBO, albeit the available literature does not show any clear evidence for this.[4]

### Detection of DNA damage

DNA-damage comprises modifications of DNA bases or damage to the backbone, such as strand breaks. Various methods for the detection of DNA damage are in use, such as [$^{32}$P]-post-labelling, alkaline unwinding, alkaline elution or the so-called 'comet assay', also known as single-cell gel electrophoresis. The comet assay detects strand breaks on the single cell level and is a simple, fast, sensitive and well-established genotoxicity test.[14,15] The comet assay is a

**Figure 1**
**Comet assay with isolated lymphocytes before and after hyperbaric oxygen (HBO) therapy**




microgel electrophoresis technique, using a small sample of cells suspended in a thin agarose gel. The sample is lysed, electrophoresed and stained with a fluorescent DNA-binding dye whilst on a microscope slide. DNA damage can be analysed by image analysis. Nuclei with increased strand breaks show increased DNA migration in the electric field, and this resembles the shape of a comet (Figure 1).

Several parameters can be quantified for determining the length and amount of DNA migration. The most frequently used parameters are 'tail intensity' (% tail DNA) and 'tail moment' (a product of both length and intensity). DNA migration is mainly induced by DNA single-strand breaks, DNA double-strand breaks and alkaline-labile sites (e.g., abasic sites). Though sensitive, the comet assay is not specific with regard to the genetic relevance of the observed effects. Different kinds of genotoxic effects can cause increased DNA migration, and further information is needed to evaluate biological significance. Such additional information can be derived by using lesion-specific endonucleases, e.g., formamidopyrimidine-DNA-glycosylase (FPG), which detects and cuts out specific oxidized bases, producing additional strand breaks.[16]

The comet assay can be performed with virtually any eukaryote cell population *in vitro* and *in vivo*. Various tissues can be comparatively investigated such as whole blood, isolated lymphocytes, liver, lung, heart and kidney. In addition, the assay is highly sensitive for even low levels of DNA damage and requires only small samples. The comet assay has already been used in many studies to assess DNA damage induced by various agents in a variety of cells *in vitro* and *in vivo*. The test has widespread application in genotoxicity testing, environmental biomonitoring and human population monitoring.

**DNA repair and mutagenesis**

The comet assay not only detects DNA damage but also enables an investigation of DNA repair. For this purpose, the time-dependent removal of induced lesions, i.e., the decrease in DNA migration, is monitored. Using the comet assay, HBO-induced DNA migration in healthy young male volunteers, who had been exposed to a therapeutic HBO treatment protocol (253 kPa for a total of three 20-minute periods of pure $O_2$ breathing, interspersed with five-minute periods of air breathing), was shown to be reduced by more than 50% within the first hour after exposure. However, it also showed that blood taken six or 24 hours after HBO no longer showed increased migration, indicating fast and complete repair of the HBO-related DNA damage.[17]

It must be noted that the comet assay measures only the kinetics of strand break repair but not its accuracy. Thus incorrectly repaired lesions do not contribute to migration but may still have a mutagenic potential. Therefore, in order to investigate the biological significance of the effects of HBO shown with the comet assay in humans, the micronucleus test (MNT) was performed. The same blood samples that showed a significant rise in DNA migration in the comet assay did not exhibit increased micronucleus frequencies.[17] Although the effects in the MNT are limited to proliferating lymphocytes, this observation demonstrates that the genotoxic effects occur in the whole population

of white blood cells. Therefore, it can be presumed that, under therapeutic exposure conditions, the primary DNA damage is repaired before the cells enter the mitotic S-phase and chromosome aberrations can be produced. In fact, no evidence of the induction of chromosome damage was found after this single *in vivo* HBO exposure in lymphocytes from healthy human volunteers.[17]

Earlier investigations suggested increased frequencies of chromosome aberrations after HBO exposure. However, these results were found in patients with diverse diseases and drug treatments after repeated HBO exposures.[18] As the comet assay results indicate that repetitive HBO exposures do not induce further DNA damage, but rather induce adaptive protection, it is likely that the observed increased chromosome aberration rates are not directly associated with the HBO treatment.

In healthy organisms, efficient antioxidant mechanisms together with DNA repair maintain a steady-state level of damage with a minimal risk to the cell or the whole organism.[29] On the other hand, we cannot exclude HBO, on comet assay results alone, as an important cause of mutational burden in situations where antioxidant defence is deficient or overwhelmed.[18]

The use of cultured mammalian and human cells *in vitro* makes it feasible to increase HBO exposure in order to investigate the question whether HBO induces DNA damage under conditions where antioxidant capacities are overwhelmed.[19] The main advantages of these *in vitro* studies in comparison to *in vivo* exposure of human subjects are the possibility of a permanent $O_2$ exposure (i.e., without interspersed air breathing) and increased pressures. Using the comet assay, a genotoxic effect of HBO could be demonstrated in diverse cell types.[19] More intense HBO exposures using both higher pressure and longer duration than for therapeutic use of HBO clearly caused mutagenic effects in cultured mammalian cells *in vitro*.[20,21] A correlation between $O_2$ partial pressure, exposure time and the frequency of chromosome aberrations was found in V79 cells (a permanent Chinese hamster cell line) using the MNT.[20,21] The clastogenic (chromosome-breaking) effect of the treatment in this cell line was directly related to the rise in DNA damage assessed with the comet assay. Increased HBO exposure also elevated mutant frequencies in a mammalian cell gene mutation assay at the tk-locus of mouse lymphoma cells.

In contrast to this, HBO failed to provoke mutations in the *in vitro* hypoxanthin-P-ribosyl-transferase test (HPRT test) with V79 cells (which mainly detects point mutations).[20,21] This negative finding suggests that, even under intensive exposure conditions, HBO does not significantly produce point mutations but mainly acts via a clastogenic mechanism. Consequently it is likely that after HBO exposure, reactive oxygen species develop their mutagenic potential through DNA lesions like single- and double-strand breaks, with gross deletions and chromosomal effects following, as a result of incomplete and incorrect repair. This clastogenic mechanism has also been proposed for normobaric hyperoxia, which induced comparable mutagenic effects *in vitro*. Taken together, the *in vitro* studies clearly prove that HBO with long exposure times or high pressure has the potential to induce mutations via a clastogenic mechanism.

One of the crucial mediators of HBO-induced DNA damage seems to be nitric oxide (NO). The release of NO is tightly regulated by the protein heme oxygenase-1 (HO-1) and an increased formation of NO *per se* caused DNA strand breaks no matter whether NO release was a result of administration of NO donors like molsidomine or due to cytokine stimulation.[22–25] The genotoxic properties of NO are presumed to be caused by the generation of peroxynitrite from NO and superoxide under conditions associated with increased release of these two molecules.[26] On the other hand, elevated DNA damage observed in other studies was not related to the blood nitrate concentrations.[27,28] It has also been noted that NO has both anti- and pro-oxidant properties depending on the local milieu, and both increased and decreased NO production has been reported during HBO exposure.[29,30]

**Protection against HBO-induced DNA damage**

As mentioned above, HO-1 plays an important role in protection against oxidative DNA damage. Lymphocytes from healthy volunteers showed significantly increased HO-1 concentration after HBO exposure both *in vivo* and *in vitro*.[31,33] Moreover, HO-1 over-expression significantly reduced the HBO-induced DNA damage in V79 cells *in vitro,*[33] whereas the inhibition of HO-1 with sn-mesoporphyrine aggravated the HBO-related genotoxicity and completely reversed the adaptive protection against HBO-induced DNA damage, again both *in vitro* and *in* vivo.[22,32,34]

The typical therapeutic HBO regimen comprises repeated HBO treatment over several days. Because it was found that a single HBO treatment induced DNA damage in healthy volunteers,[35] and there is the already mentioned close dose-effect relationship concerning both duration and pressure,[20] it was supposed that repetitive therapeutic HBO treatments may lead to a significant accumulation of DNA damage which might cause a significant mutagenic risk. However, it has been shown that human volunteers undergoing repeated HBO exposures exhibited DNA damage only after the first treatment, but not after any subsequent exposure.[17,36] In fact, the number of DNA strand breaks after repeated HBO exposures was even lower than in the initial blood sample taken before the first HBO.[17,36]

Another interesting finding is the fact that a lower initial dose of HBO (20 min at 153 kPa (1.5 bar)) did not induce any

DNA strand breaks but was associated with the induction of adaptive mechanisms that protected against further HBO-induced DNA damage.[17,36] Subsequent studies have shown that the adaptive effect is due to a cellular response that cannot be explained by enhanced repair activity and seems to be a consequence of either increased scavenging of oxygen species distant from nuclear DNA or enhanced sequestration of transition metals.[17,36]

**The role of antioxidants for DNA protection**

In the course of evolution, oxygen-consuming organisms have developed a variety of defence mechanisms against oxidative stress. Several enzymes show strong antioxidative properties, e.g., superoxide dismutase (SOD), which catalyses the dysmutation reaction of the superoxide radical.[5,37] The product of this reaction is $H_2O_2$, which in turn is either catalysed into water and molecular oxygen by the enzyme catalase, or removed by glutathione peroxidase (GPx).[5,38] GPx catalyses the reaction of two molecules of reduced glutathion (GSH) and $H_2O_2$ to the oxidized form GSSG and two molecules of water.[5,9,39]

Besides enzymes, vitamins (e.g., vitamin E and C) play an important antioxidative role.[38–40] Vitamin C has two functions. Firstly, it is needed to restore Vitamin E located in lipoproteins and membranes, where it interrupts the radical-induced chain reaction of the lipid peroxidation, and, secondly, Vitamin C has radical scavenging properties of its own. Whether exogenous antioxidant supplementation prevents HBO-induced genotoxicity is still a matter of debate. Vitamin E and the synthetic antioxidant N-acetylcysteine did not affect the HBO-induced DNA damage in healthy volunteers,[40] but no data are available in patients with decreased antioxidant capacity. However, N-acetylcysteine attenuated the rise of blood lipid peroxidation markers in patients undergoing repetitive HBO treatment sessions.[41] Glisodin®, an orally effective nutritional formula containing a plant (*Cucumis melo L.C*) SOD extract, effectively protected white-blood-cell DNA against formation of strand breaks in healthy volunteers.[42] These results indicate that long-term prophylactic antioxidant supplementation may indeed attenuate HBO-induced DNA damage.

**Diving and DNA damage**

Given the well-established phenomenon of HBO-induced DNA damage one might assume that frequent diving might also influence DNA damage, either due to a possible induction of protective adaptive mechanisms or as a consequence of an increased sensitivity against increased oxygen partial pressure ($ppO_2$). Interestingly in a yet unpublished study with healthy male recreational scuba divers of at least four years' experience, including at least 50 dives per year at depths of more than 10m, our group found that isolated lymphocytes exposed to HBO (two hours, 405 kPa) did not show any difference in the induced tail moments compared to lymphocytes from non-diving volunteers of the same age. Subsequently we studied combat swimmers and underwater demolition team (UDT) divers.[43] These subjects perform dives over several years breathing pure $O_2$ and/or $O_2$-enriched inspiratory gas mixtures using closed and semi-closed breathing apparatus respectively. Thus, these divers represent a population with a particularly long-term repetitive exposure to increased $ppO_2$. Isolated lymphocytes from these groups were compared to those from both non-diving naval pentathlon athletes (chosen because they have a comparable degree of endurance training to the diver groups) and untrained controls of the same age following the same HBO regimen mentioned above. DNA repair was maintained over 2 hours after HBO exposure. As shown in Table 1 all groups showed a marked rise in the tail moment, which was, however, nearly twice as high in the combat swimmers as in the three other study groups. Nevertheless, in all groups, the increased tail moment returned to normal values within one hour after the HBO exposure, without any inter-group difference. Hence, combat swimmers who undergo particularly high and prolonged HBO exposures not only show the most pronounced HBO-induced DNA damage but also the most rapid and effective repair.[43]

**Conclusion**

The DNA damaging and mutagenic potential of HBO is not in dispute, as shown by *in vitro* studies with mammalian cells. DNA damage has been observed with therapeutic exposures, but mutations and chromosome aberrations were not detectable in blood cells under the same conditions.

**Table 1**
**DNA-damage in isolated and HBO-exposed lymphocytes after long-term, repetitive exposure to increased $ppO_2$; data are mean (standard deviation); * depicts P < 0.05 versus control; § depicts P < 0.05 versus pre-HBO (from reference 43, with permission)**

| | pre HBO | post HBO | 1 h incubation | 2 h incubation |
|---|---|---|---|---|
| **Combat swimmers** (N = 7) | 0.12 (0.03) | 0.38 (0.09)*§ | 0.13 (0.04) | 0.10 (0.01) |
| **UDT divers** (N =7) | 0.10 (0.02) | 0.24 (0.08)§ | 0.12 (0.03) | 0.10 (0.02) |
| **Navy pentathlon athletes** (N = 6) | 0.10 (0.02) | 0.22 (0.05)§ | 0.10 (0.01) | 0.11 (0.02) |
| **Control** (N = 24) | 0.12 (0.04) | 0.28 (0.14)§ | 0.13 (0.04) | 0.14 (0.05) |

Even if blood cells do not seem to be subject to an increased risk of (chromosome) mutations, mutagenic effects in other target cells cannot be completely excluded and, hence, the potential genotoxicity of hyperbaric oxygen should be taken seriously.

On the other hand, there is a fast repair of oxidative DNA damage as well as an adaptation to subsequent oxidative stress. Furthermore, a simple and efficient way to prevent organisms from HBO-induced DNA damage is to start with a shortened treatment before the standard protocol is applied, and therefore an adaptation of the commonly used treatment protocols should be considered.

The use of antioxidants such as vitamin C, E or even N-acetylcysteine seems to be ineffective in preventing HBO-induced genotoxicity. In contrast, the orally effective vegetal SOD (Glisodin®) protected against HBO-induced DNA damage and thus may play a role in the prevention of such damage. Finally it has to be pointed out that, regarding the effect of antioxidants, the available data refer to collectives of healthy and young volunteers, and no firm conclusions can be drawn for patients with a reduced antioxidative capacity (e.g., radionecrosis, chronic wound healing defects, chronic infection).

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