# PAPERS FROM THE JOINT SPUMS AND ROYAL HOBART HOSPITAL MEETING, NOVEMBER 1988

## OXYGEN PRODUCED FREE RADICALS

Christian Narkowicz

This is a quick resume of oxygen produced free radicals because what one really does in the chamber is expose people to high concentrations of oxygen. Oxygen is known to be toxic. At least some of this toxicity is due to the radicals formed which can include superoxide which will dismute to hydrogen peroxide. Hydrogen peroxide can be removed by disproportionation catalysed by catalase or through peroxidation with various peroxidases. However if all the hydrogen peroxide is not eliminated and there is $Fe^{++}$ or $Cu^{+}$ present a highly reactive hydroxyl radical is formed which is potentially very damaging. What we wanted to know was whether hyperbaric oxygen resulted in an increase of oxygen radicals, or free radicals, and whether the body was able to cope with increased free radical levels, whether we actually do damage to the patients that we are trying to treat.

We have been very lucky to get the assistance of the Tasmanian Police divers. They have given us great co-operation and without them we could not have done these studies. In all we put 10 Tasmanian police divers into our chamber. We gave them 3 consecutive 20 minute periods of breathing oxygen at 3 ATA and we took a venous blood sample after each of these periods, and a final sample back at the surface. We froze the blood samples in liquid nitrogen, and analysed them for free radical levels by ESR, which stands for electron spin resonance. The unpaired electron in a free radical has magnetic properties and one can exploit these magnetic properties to directly measure free radical levels.

To perform an ESR experiment we put the sample between the poles of a very strong magnet that polarises the unpaired electrons into a high energy level and a low energy level. We can induce transitions from the low to the high energy level by irradiating with microwaves at an appropriate frequency (figure 1) We irradiate with microwaves and sweep the magnetic field, and as we hit the resonance condition, where the difference in energy levels is equal to the energy of the microwaves, there one gets absorption. We plot the first derivative of the absorption curve versus increasing magnetic field (Figure 2). The second signal is due to copper in caeruloplasmin and if we were to extend this spectrum there would also be a signal from iron.

A typical trace from a normobaric venous sample has an absorption band, the height of which reflects the concentration of free radicals in the blood (Figure 2). After hyperbaric oxygen treatment there is a significant increase in the level of free radicals. In the post hyperbaric oxygen graph, the peak appears to be 3-4 times the size of the pre-hyperbaric oxygen. Can one make a quantitative statement about that and say there is 3-4 times as much free radical? One can. The integral of the signal is directly proportional to the free radical concentration, assuming identical running conditions for the instrument. The integral is actually proportional to the height of the signal, so in hyperbaric oxygen there were roughly 10 times the concentration of free radicals that were in the baseline data.

**FIGURE 1**

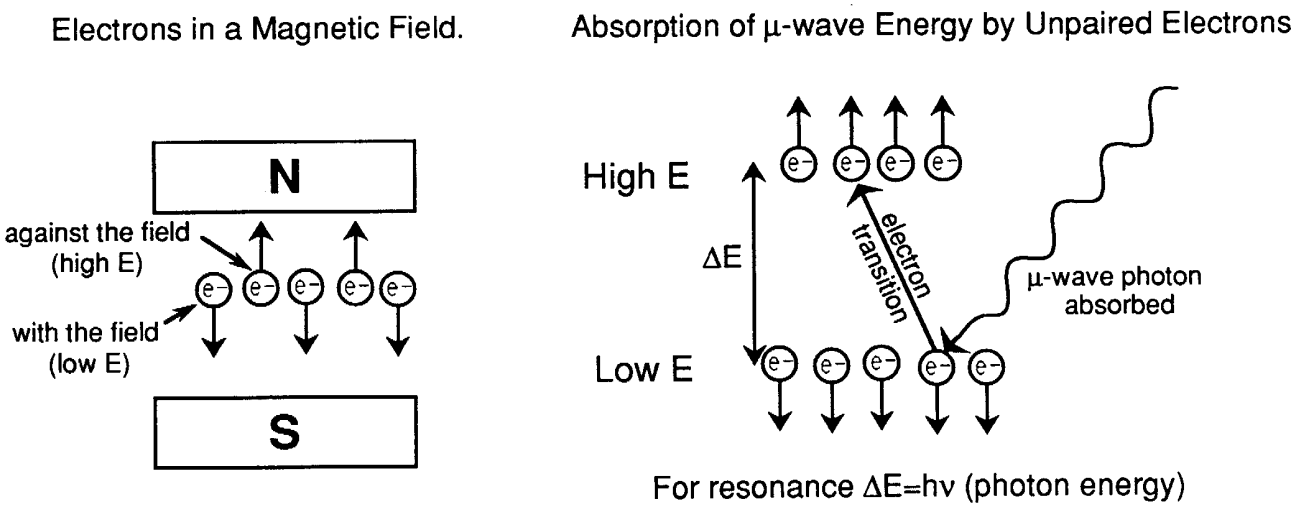


**FIGURE 2**

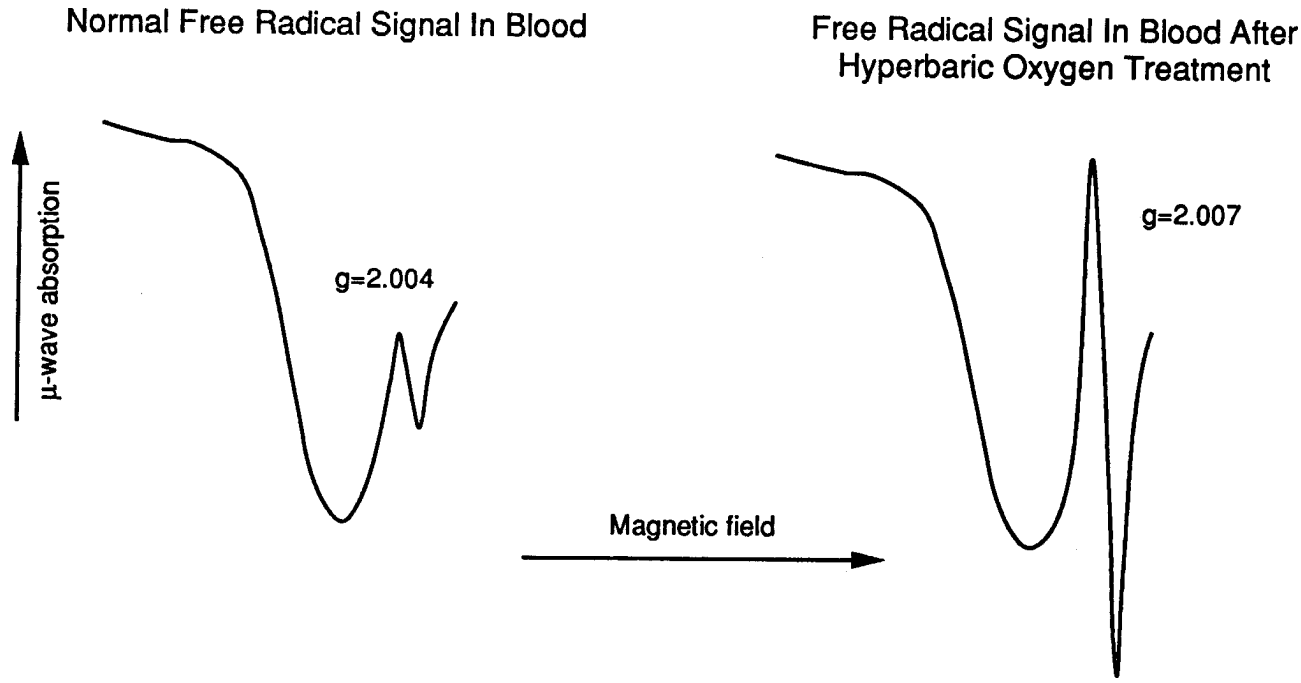



**FIGURE 3**

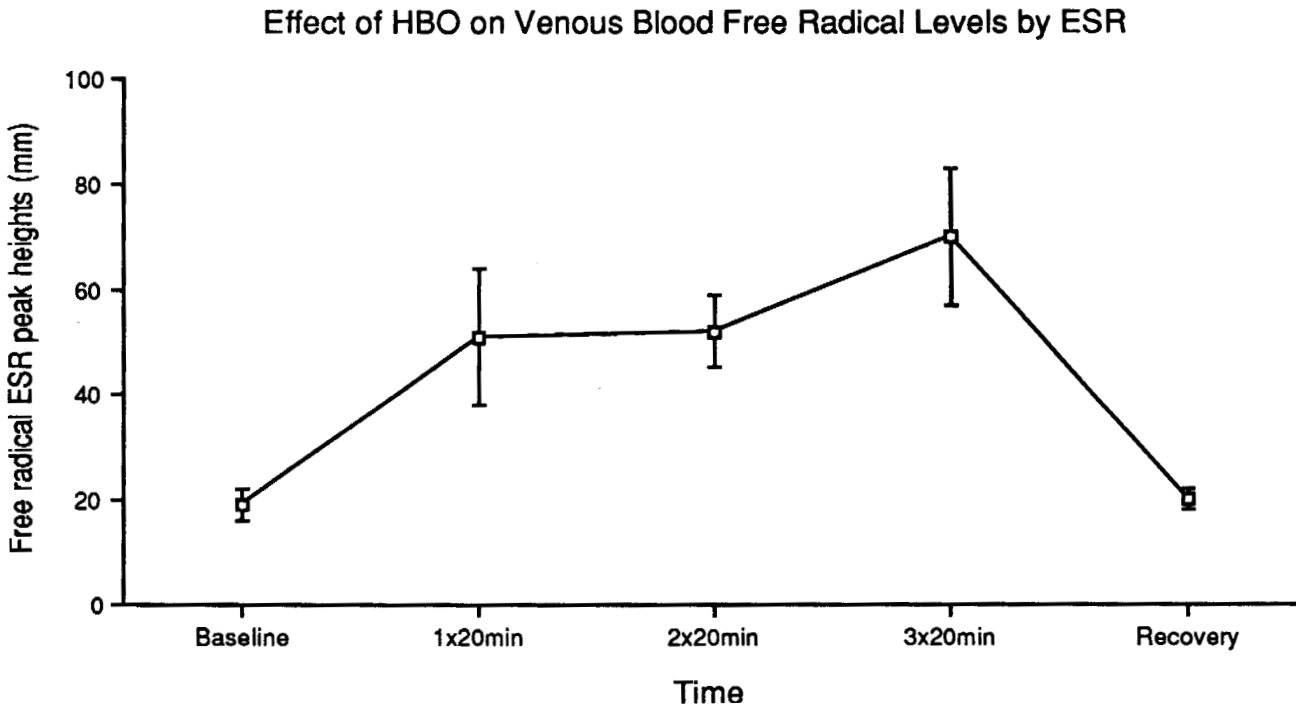
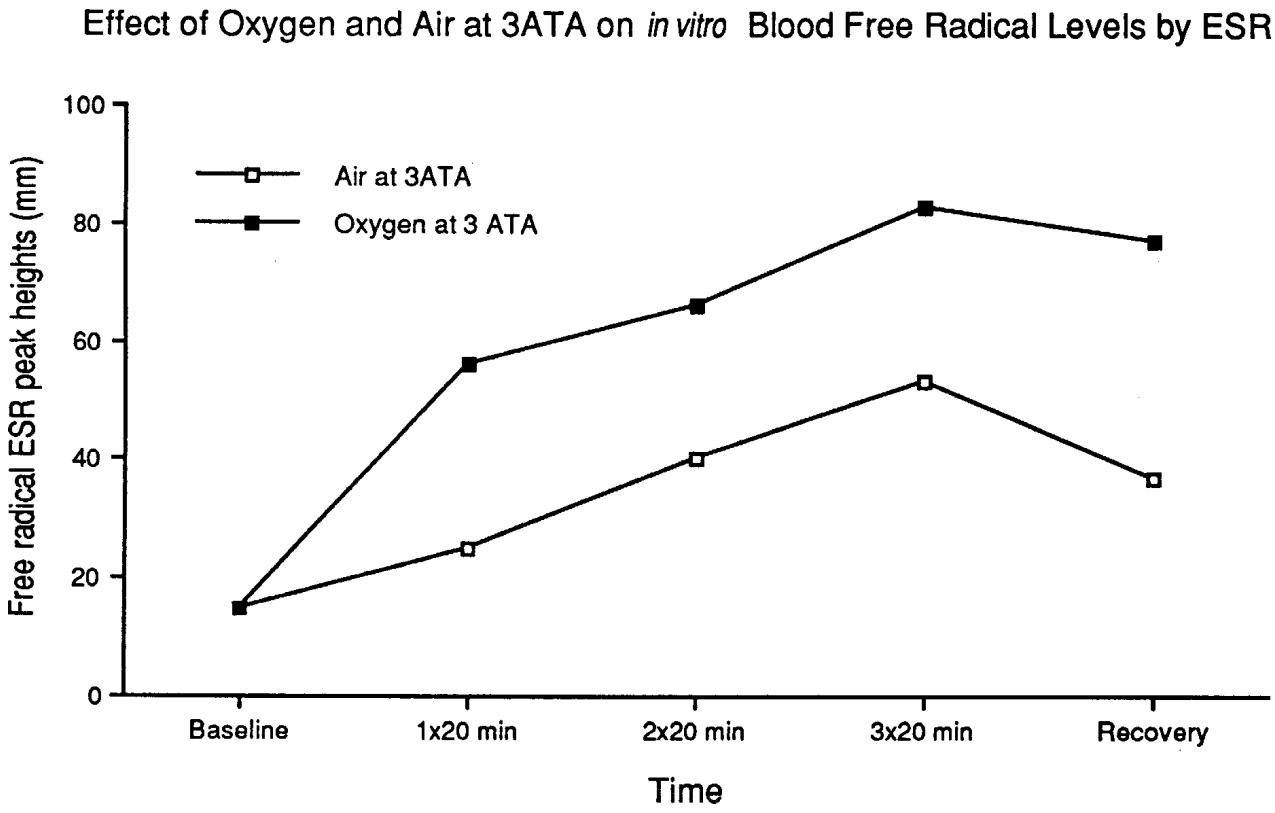


**FIGURE 4**




The average response in our 10 police volunteers after three 20 minute periods in oxygen is shown in Figure 3. On average it is around a 400% increase after 60 minutes, which is Dr Peter McCartney's normal protocol for hyperbaric oxygen therapy. Now to compare these results with an in vitro sample of blood bubbled with oxygen. If blood in vitro is bubbled with oxygen and blood in vitro bubbled with air, there is a similar sort of response (Figure 4), except one does not get the drop off at the end of the exposure which occurs in vivo because in vitro there is less oxygen consumption. So the oxygen levels remain high at the end of the run. There is some relationship between the concentration of oxygen and the degree of free radical production.

We found that the hyperbaric signal was different in form to the signal in ordinary, normobaric blood. By increasing the power of the microwaves used to obtain a spectrum one gets a linear increase in the height of the signal, with an increase in the square root of power, until one reaches a time where one starts getting a decrease in population difference between the high energy and the low energy levels, which is referred to as power saturation. The actual strength of the signal depends on that difference in population levels. If the high energy electrons can not lose energy quickly enough to make up for the energy that is coming into the system, the population difference decreases and there is a drop off in signal. We found that the hyperbaric signal dropped off a lot earlier than the normobaric signal indicating that it is a different free radical involved to the the free radical in normal blood. For free radicals it could be that several radicals contribute to each signal. The baseline free radical signal is the signal that appears under normobaric conditions and with an increase in oxygen load that signal does increase to some extent but it is a second signal which increases to the greatest extent and that tends to drop off to virtually non significant levels when breathing air after the hyperbaric treatment.

From further experiments we believe that the baseline signal may be due to semi-reduced glutathione reductase which is associated with the pentose phosphate pathway which is responsible for maintaining cells in the reduced form, for overcoming oxidative stress in cells. The baseline signal comes from the red cells. We are not sure exactly where the second signal comes from. With increasing oxygen concentrations one would expect to get increased oxidation of hemoglobin to methemoglobin with loss of superoxide, and the superoxide then has to be scavenged which requires energy for the system to be reduced back to its reduced state. That is where the pentose phosphate pathway comes in by providing the substrates for maintaining the reduced state of the cell. The second signal we have not been able to assign to a particular radical as yet but it could be due to a scavenger or perhaps associated with lipid peroxidation, though the power saturation is not consistent with being an oxygen radical nor an alkoxyl radical nor a lipid peroxyl radical.

At this stage all we can say is that exposure to limited periods of hyperbaric oxygen does cause an increased level of free radicals in blood. The healthy human appears to clear this increase rapidly on re-exposure to room air. But as yet

we have not assessed any possible damage, such as lipid peroxidation in the blood, by other chemical means. There is certainly room for further experiments.

**Edited comments from the question and answer session appear below**

Dr Ian Unsworth

This is fascinating to me. If there is a rise of radicals during a period of high pressure oxygen it would seem to suggest that the body's normal mechanisms for dealing with radicals have been overcome, and so the radicals are allowed to build up. But it also suggests that when one brings the subject back to one atmosphere radicals very quickly stop forming. Presumably this is due either to automatic dissipation of the radicals or to an extremely quick build up of the body's scavenging. Have we any idea which it might be? As the radicals fall so quickly one can hypothesise that the scavengers which have been knocked out under pressure regenerate very quickly.

C.Narkowicz

The pentose phosphate pathway will provide substrates for regeneration of the antioxidants of the free radical scavengers. The signal that we see could actually be a free radical scavenger because they exist as a free radical once they have scavenged the radical. They take the free radical character from the scavenged radical and become radicals themselves, so it could be something like vitamin E which is scavenging and as yet has not been reduced via the ascorbate pathway and NADPH (nicotinamide-adenine dinucleotide phosphate [reduced form]) back to the reduced form. It will take further experiments, perhaps with our own spin trapping agents which, so to speak, interact with the radicals as they are produced, and trap the radicals in a stable form before they can be reduced by other antioxidants. Spin traps can enable one to identify the actual structure of the radical being formed.

Dr P. Chapman-Smith

Policemen remain fairly docile and blood is easy to get at. Have you any comments on tissue free radicals? Obviously it is a lot harder to chip bits out of the local policeman.

C.Narkowicz

There has been a lot of work on radicals in tissue but not as far as I know concerned with hyperbaric oxygen. They certainly found increased levels of radicals under certain pathological conditions and after exposure to various carcinogens and drugs. Various drugs increases the free radical levels in the tissue. As for our experiments I guess one would have to get a pig or a sheep into the chamber, or a rat.

Dr Janet Vial

There are some problems with looking at tissue samples with ESR. The problem that bedevils this area is artifacts, especially when you are using ESR to measure tissue radicals. Just cutting tissue in itself can generate radicals. One has to be very careful about the way the tissues are handled. It is obviously important that it be frozen immediately to lessen this problem of artifact radicals.

Dr David Davies

Have you pushed your times under pressure any further than 60 minutes? Do you plan to?

C.Narkowicz

We have not gone beyond 60 minutes. To go longer is up to Dr Peter McCartney, and up to the policemen too. Certainly treatments go beyond 60 minutes. For instance Dr Ian Unsworth is treating carbon monoxide poisoning with 90 minutes of 100% oxygen at 2.4 atmospheres. It would be interesting to see whether there was any further increase in free radicals at the end of that 90 minutes.

There is a possibility that there is a point at which one overwhelms the body's own defences and one will get a massive increase in the free radical levels, but that could be 60 minutes, 90 minutes, or it could be 2 hours. We really do not know. It will also depend on the individuals and their diet and fitness and age, etc. It is certainly something worth doing. It would be interesting to see what happens at different exposures.

Dr David Davies

Another thing to consider is doing something similar on saturation divers at pressure. They can be at 200 m pressure for 3 weeks.

C.Narkowicz

However they have their oxygen supply diminished proportionally. But it might show whether it is an effect of pressure or an effect of oxygen. It does seem to be an effect of oxygen rather than of pressure because if we use nitrogen in vitro there is not the increase that occurs with oxygen. Of course we can not use only nitrogen with our police divers, nor with abalone divers.

Divers, from my experience anyway, seem to age quickly. I do not know if this is backed up by statistics but they seem to have a lot of medical problems, arthritis, and premature aging. I do not know if it is their very hard lifestyle or drinking and smoking.

Dr Chris Lourey

Apart from the incidence of osteonecrosis the punch drunk diver abalone diver is no different from the punch drunk crayfisherman. I think it reflects their after aquatic lifestyle and probably their alcohol intake rather than their diving.

C.Narkowicz

There is certainly an increase in free radicals just with breathing air. So at 18 m on air one is also increasing the oxidative stress.

Dr Peter Chapman-Smith

Are there specific tissues that have been shown to be damaged by this release of free radicals?

C.Narkowicz

The eyes are very susceptible. The retrolental fibroplasia of premature infants is caused by oxygen. The red cells are very vulnerable simply because they carry a lot of the oxygen and actually produce a lot of the radicals. As for other tissues, brain and marrow and the lungs.

One of the problems is that most of the evidence so far is circumstantial. We can show experimentally that if one gives free radical protecting agents one can reduce the damage but most of the evidence is circumstantial because of the difficulty of actually measuring free radicals in these situations and of showing that there is an increase in free radicals associated with the damage.

It would be interesting to put someone with a 6-glucose phosphate dehydrogenase deficiency into the chamber and see what happens to them. They have a slower rate of free radical inactivation, with a decreased pentose phosphate metabolism. So they might be suceptible to oxidative stress a bit earlier than someone with a fully functional pentose phosphate pathway.

Question

Does microwaved food generate free radicals?

C.Narkowicz

Not that I know of. I do not think it should but food irradiation is known to produce to hydroxyl radical which is where a lot of the concern comes from because if those radicals are not scavenged by the antioxidants in the food they can affect the proteins, the vitamins, and really affect the quality of the food. Another thing is that ultrasound is known to produce hydroxyl radicals as well. So there is increasing concern over use of ultrasound, or the excessive use of ultrasound, and of course exposure to radiation. Most of the damage is by free radical mechanisms.

*This is an edited transcript of a recording made at a Free Radical Workshop during the joint SPUMS and Royal Hobart Hospital meeting on Hyperbaric and Diving Medicine in November 1988.*

*Mr Christian Narkowicz, B.Sc.(Hons) is attached to the Department of Medicine, University of Tasmania Clinical School, 43 Collins Street, Hobart, Tasmania 7000, Australia.*

## IRON, OXYGEN RADICALS AND THE JOINT

Fiona Andrews

Iron has been associated with joint inflammation for a number of years. As long ago as 1674 Hochsletter described an arthritis associated with excessive bleeding in haemophilia patients. This was reproduced in experimental animals by injecting blood into the joints. Later on it was also shown that patients with oral iron overload or haemochromatosis also had iron deposits within the synovial membrane, and this was associated with inflammation of an otherwise normal joint.

Interest in iron and joint inflammation was rekindled by the work of Muirden[1] who showed that iron deposition occurred in the synovial membranes of patients with rheumatoid arthritis. It was speculated that perhaps these iron deposits had some role in the pathogenesis of the disease. This leads us to the question, how does iron deposition arise in the rheumatoid joint?

In normal situations iron is carefully conserved and recycled within the body and very little is lost. But in chronic inflammatory conditions such as rheumatoid arthritis many patients become anaemic. This does not appear to be due to an increased loss of iron but to a sequestering of iron within the reticuloendothelial cells so the iron is not let out back into the recirculating pool. How does this relate to the joint? Ultrastructural studies have shown that the synovium consists of reticuloendothelial like cells and it was proposed that iron deposits, derived from periods of micro bleeding in the joint are sequestered in the reticuloendothelial-like cells of the synovium. In support of this Muirden[2] found that levels of iron within the synovium correlated with an increased activity of joint inflammation.

So how might iron enhance inflammation in the rheumatoid joints? One theory is that iron is involved in oxidative tissue damage. The environment of the inflamed rheumatoid joint is highly suited to the production of reactive oxygen metabolites such as superoxide, hydrogen peroxide and the hydroxyl radical. The major source of reactive oxygen metabolites are the infiltrating inflammatory phagocytic cells. Phagocytosis induces increased cellular aerobic activity which leads to the formation of relatively unreactive superoxide and water. In the presence of iron however hydroxyl radicals, the most toxic reactive oxygen metabolites can be produced. A further factor that leads to an ideal environment for reactive oxygen metabolite activity is an insufficient reactive oxygen metabolites scavenging ability which has also been demonstrated in the rheumatoid joint.

What evidence have we that reactive oxygen metabolite reactions are occurring in the joint? Several studies have demonstrated the presence of lipid peroxidation prod-