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MYOCARDIAL TISSUE PREPARATION FOR ESR SPECTROSCOPY: SOME METHODS MAY CAUSE ARTIFACTUAL GENERATION OF SIGNALS

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It has been suggested that some techniques of tissue preparation for esr spectroscopy may artifactually generate radicals. We have investigated this, together with the possibility that the susceptibility of the tissue to preparation artifacts may be altered by ischaemia and reperfusion. Three different methods of tissue processing have been assessed: (i) freeze-clamping (−196 °C), using grooved, aluminium tongs which produce frozen cylinders of tissue (3 mm diameter) which fit directly into esr tubes; (ii) grinding of freeze-clamped tissue with a porcelain pestle and mortar; (iii) lyophilisation of ground, freeze-clamped, tissue. Isolated rat hearts ($n = 7$ or $n = 5$/group) were subjected to aerobic perfusion (10 min, 37 °C), total, global ischaemia (15 min) and reperfusion (30 sec). Hearts were freeze-clamped at the end of each period. Tissue was prepared by each of the three methods and esr spectra recorded at −100 °C. In spectra from tissue which had been freeze-clamped only, broad high- and low-spin iron III signals ($g = 1.9$, $g = 2.2$–2.9 and $g = 4.6$) were seen together with a narrow, well-defined signal ($g = 2.005$), possibly from a semiquinone radical. In spectra from ground samples, an anisotropic signal ($g_{\parallel} = 2.040$ and $g_{\perp} = 2.008$), probably from a peroxyl radical, was observed in addition to the iron III signals. The intensity of the anisotropic signal varied with perfusion conditions; in ischaemic tissue it was decreased to 33 ± 10% of the control value and in reperfused tissue it was decreased to 76 ± 26%. In spectra from lyophilised samples, a narrow signal ($g = 2.009$), probably from a protein radical, was observed in addition to the iron III signals. The intensity of the signal at $g = 2.009$ was increased in ischaemic tissue to 170 ± 57% of the control value and in reperfused tissue to 241 ± 85%. In conclusion, artifactual generation of radicals can occur upon grinding (peroxyl radical) and lyophilisation (protein radical). Ischaemia and reperfusion may alter not only radical content per se but may also modify the susceptibility of the tissue to the artifactual production of radicals.

KEY WORDS: Free radicals, tissue processing, artifacts, esr spectroscopy.

1. INTRODUCTION

It has been suggested that the pathological events which occur as a result of myocardial ischaemia and reperfusion are triggered, at least in part, by cytotoxic active oxygen species such as superoxide anion radicals, hydroxyl radicals[1,2] and singlet

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oxygen.[3,4] Until recently, much of the evidence to support this hypothesis has been indirect and has depended on the use of antioxidant enzymes, free radical scavengers and quenchers of singlet oxygen.[5-11]

Several recent direct studies, using electron spin resonance (esr) spectroscopy, have confirmed that free radicals are generated during the first few minutes of reperfusion of the ischaemic rat, rabbit and dog myocardium. Using the technique of spin trapping, oxygen- and carbon-centred radical adducts have been observed and quantified in the effluent of isolated, reperfused, rat hearts,[12-14] oxygen radical adducts have been detected in the effluent from isolated rabbit hearts,[15] and carbon-centred radical adducts have been detected in the blood from dog hearts reperfused *in vivo*.[16] Free radicals have also been detected in heart tissue following reperfusion,[17] but because of the short half-life of reactive free radicals, it is necessary to work with rapidly frozen tissue. The frozen heart tissue can then be treated in order to introduce it into the esr cavity for spectroscopic measurements; for example, lyophilisation, grinding or fracturing. It is well known that procedures such as lyophilisation,[18] grinding[19,20] and cutting[21,22] may affect the free radical spectrum observed with frozen tissue. Such considerations raise into question the best approach to the study of free radical changes in reperfused heart tissue. Of the various methods available, rapid freezing using pre-cooled clamps with specially designed cylindrical grooves which facilitate direct usage of the frozen cylinders of heart, was selected as being the procedure least likely to generate mechanically created artefacts. In the present study, this method has been compared with those of lyophilisation and grinding in order to assess the extent of artifactual free radical production in samples from aerobic, ischaemic and reperfused heart tissue.

2. METHODS

A. *Animals*

Male Wistar rats (250–300 g body weight) were used in all studies.

B. *Perfusion Techniques and Perfusion Media*

Rats were lightly anaesthetised with diethyl ether and after the administration (i.v) of heparin (200 IU), hearts were excised and aerobically perfused[23] (95% O_2 + 5% CO_2), at a constant pressure of 65 cm H_2O at 37 °C. The perfusion medium was bicarbonate buffer containing (in mM) NaCl (118.5), $NaHCO_3$ (25.0), KCl (3.1), $MgSO_4$ (1.2), KH_2PO_4 (1.2), $CaCl_2$ (2.5) and glucose (11.1); it was passed through a 0.22 μm filter prior to use.

C. *Tissue Processing*

Hearts ($n = 7$ or $n = 5$/group) were perfused aerobically for 10 minutes, followed by total, global ischaemia for 15 minutes, achieved by clamping the aortic input line, and aerobic reperfusion for 30 seconds. At the end of each period (aerobic, ischaemic and reperfusion), hearts were freeze-clamped using specially constructed aluminium tongs (Biomedix, Pinner HA5 5BY, UK; see Figure 1), cooled to − 196 °C. The tongs had a series of parallel grooves (3 mm wide and 50–67 mm long) milled into one face such that upon freeze-clamping, the heart tissue was compressed into cylinders which were

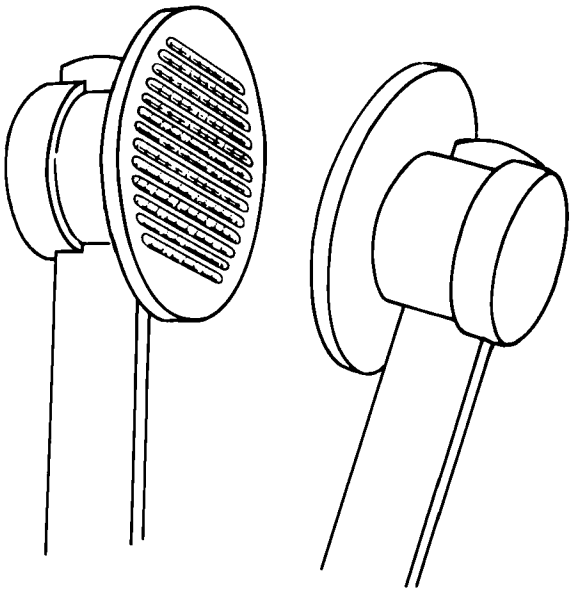

FIGURE 1 Diagram showing the specially constructed freeze-clamps used in this study (Biomedix, Pinner, UK).

of the correct dimensions to fit directly into esr tubes of 3 mm internal diameter without the need for further manipulation or processing (first group). In the second group, the freeze-clamped cylinders of tissue were ground under liquid nitrogen using a porcelain pestle and mortar and the resultant fine, frozen powder used to fill esr tubes to a depth of approximately 30 mm. In a third group, the ground tissue was lyophilised and esr tubes were then filled with the dried powder. Spectra were recorded from all three groups of tissue at $-100\,^{\circ}C$. To standardise conditions, the tissue grinding was always carried out by the same person for a measured time period (2 minutes) using similar quantities of tissue and the same apparatus.

D. *Esr Spectroscopy*

All esr spectra were recorded under identical conditions at $-100\,^{\circ}C$ using a Bruker ER 200D or a Bruker ESP 300 spectrometer; previous studies have shown that there is no significant difference in the esr response at $-100\,^{\circ}C$ compared with $-196\,^{\circ}C$ (see also[20]). Both machines were equipped with 100 KHz modulation and a Bruker ER 411 variable temperature unit. Relative radical concentrations were measured from the peak to peak signal heights and g-values were calculated relative to a standard sample of the diphenyldipicrylhydrazylradical (DPPH).

E. *Tests of Statistical Significance*

Results were expressed as the mean $\pm$ SEM. Each group was compared to the control using the unpaired t-test and Dunnett's correction. P values of less than 0.05 were considered to be statistically significant.

3. RESULTS

A. *Freeze-clamped Tissue*

In wide scans (5000 G) of aerobic tissue, a number of very broad peaks at g = 1.9, 2.2–2.6 and 4.6 were visible (Figure 2). Due to the particular combination of *g*-values observed, these signals are assigned to low- and high-spin iron III haem systems such as those found in the ferrihaemoproteins haemoglobin, myoglobin and cytochrome c.[24] The intensities of these iron signals were similar in all spectra from aerobic, ischaemic and reperfused tissue. The paramagnetic components in the frozen tissue that were associated with the signal absorptions shown in Figure 2, were not saturated by increasing microwave power up to 100 mW. This behaviour is characteristic of metal-dependent systems.[25]

Figure 3A shows a typical esr spectrum (narrow scan, 200 G) obtained from aerobic tissue. The spectrum consists of a narrow, isotropic signal at $g = 2.005$ (line width = 9.2 ± 0.1 G). In contrast to the iron signals, this isotropic signal was influenced by both ischaemia and reperfusion, and was easily saturated by increasing the microwave power. Samples from ischaemic tissue gave spectra in which the signal was greater in height and narrower than in the aerobic controls (line width = 8.0 ± 0.1 G, $p < 0.001$ compared to the aerobic controls). Samples from reperfused tissue gave spectra in which the signal was similar in height to aerobic controls, but was broader (line width = 10.0 ± 0.1 G, $p < 0.001$ compared to the aerobic controls). This isotropic peak is characteristic of a semiquinone radical, possibly ubisemiquinone (coenzyme *Q*), a relatively stable free radical which is involved in oxidative phosphorylation.[26,27]

B. *Freeze-Clamped and Ground Tissue*

In spectra from ground, aerobic tissue, in addition to the broad iron III peaks (spectra not shown) seen in freeze-clamped tissue, an anisotropic peak was seen with com-

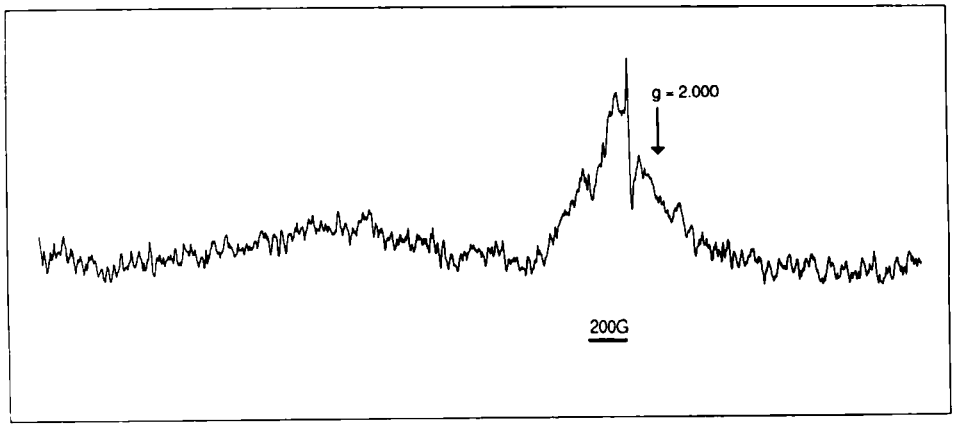

FIGURE 2 Typical electron spin resonance spectrum of freeze-clamped, aerobic tissue showing peaks assigned to high- and low-spin iron. Spectrometer conditions: modulation amplitude 2.0 G, time constant 2.0 s, scan time 2000 s, field 2250 G, scan 5000 G, power 20 mW, frequency 9.48 GHz.

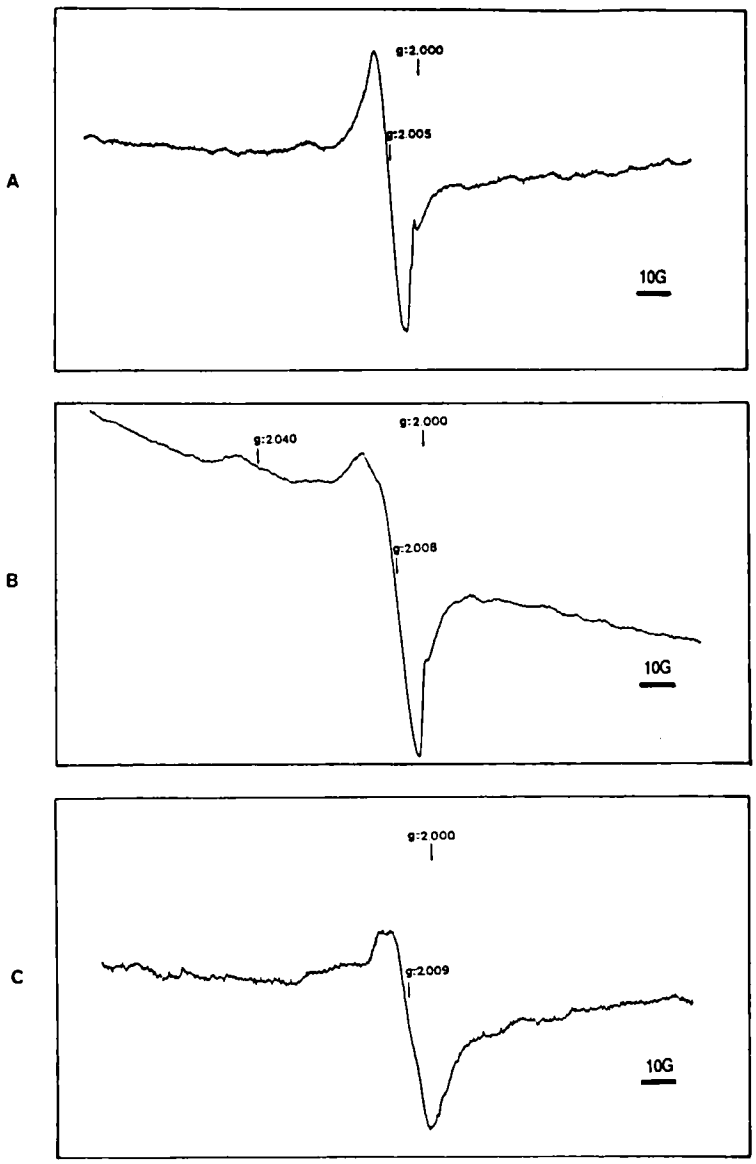

FIGURE 3 Typical electron spin resonance spectra from: (A) Freeze-clamped tissue (gain 4×10^6), (B) Ground, freeze-clamped tissue (gain 2×10^6), (C) lyophilised, ground and freeze-clamped tissue (gain 4×10^5). All spectra are from aerobic tissue. Spectrometer conditions: modulation amplitude 2.0 G, time constant 2.0 s, scan time 2000 s, field 3330 G, scan 200 G, power 20 mW, frequency 9.48 GHz.

ponents at g 2.040 and $g_\perp = 2.008$ (Figure 3B). The g-value, response to increasing microwave power, shape and anisotropic character of this signal are comparable to those of organic peroxyl radicals[20,25] and we believe that the radical species involved is probably a polyunsaturated fatty acid peroxyl radical, possibly arising as a conse-

quence of lipid peroxidation, induced as a result of the tissue disruption incurred during the grinding process. Alternatively, the peroxyl radical signal may have arisen as a result of the disruption of an iron-dependent enzyme system such as cyclooxygenase or lipoxygenase.[20] The signal height varied with perfusion conditions; in ischaemic tissue it was decreased to 33 $\pm$ 10% of the aerobic value ($p < 0.01$; $n = 7$) and in reperfused tissue, it was decreased to 76 $\pm$ 26% of the aerobic value ($p < 0.01$; $n = 7$). The $g = 2.005$ signal seen in spectra from freeze-clamped tissue may have been present, but it would have been obscured by the anisotropic signal.

C. *Freeze-clamped, Ground and Lyophilised Tissue*

In addition to the broad iron III peaks seen in freeze-clamped tissue, an isotropic signal ($g = 2.009$, line width = 8.7 $\pm$ 0.4 G) was observed in spectra from lyophilised, aerobic tissue (Figure 3C). The flattening of the low field part of the signal may be due to fine splitting of a single radical or to a mixture of unresolvable radicals. The signal height varied with perfusion conditions; in ischaemic tissue it was increased to 170 $\pm$ 57% of the aerobic value ($p < 0.025$; $n = 5$), and in reperfused tissue it was increased to 241 $\pm$ 85% of the aerobic value ($p < 0.025$; n = 5). The line width of the $g = 2.009$ signal was not influenced by the perfusion conditions; in ischaemic tissue, the line width was 8.0 $\pm$ 0.6 G and in reperfused tissue, the line width was 8.4 $\pm$ 0.6 G.

4. DISCUSSION

A. *ESR and the Problems of Sample Preparation*

The recent growth of interest in the possibility that free radicals play a role in the pathophysiology of ischaemia-induced and particularly reperfusion-induced injury has focussed attention on the need to detect and quantify free radicals and to show that their activity is limited by various "anti-free radical" interventions.[6] As we have stressed before,[12] in most studies of cardiac injury, the association between free radicals and tissue damage is indirect and as such, may be circumstantial.

The routine use of esr for the direct measurement of radicals in biological samples is hampered by the severe constraint put upon the tissue sample being measured by the microwave absorption of water at the commonly used esr frequency of 9 GHz (X-band),[27] which makes it necessary to minimise the effective water content of any sample before esr measurements can be made. This problem can be approached in a number of ways: by removing the water from the sample completely (by lyophilisation); by decreasing the amount of water present in the cavity (by using a flat cell); or by changing the water to a less absorptive state (by freezing the sample). This difficulty, inherent in esr studies of biological tissue, is compounded by the size of the esr cavity in commonly available instruments, which limits the sample tube size to 4 mm OD. To date, a number of methods, all of which involve the processing of frozen tissue (at $-$ 196 °C), have been used to comply with these constraints. These include grinding with a pestle and mortar,[20,28] pulverisation using a percussion mortar,[17,30] chopping or cutting into small fragments,[29,30] or lyophilisation.[18] All of these methods involve considerable sample disruption and it has been suggested that each method can artifactually generate radicals.[18,20–22,25,28,29]

In the present study, we have used specially constructed freeze-clamps to produce frozen cylinders of tissue which fit directly into esr sample tubes. This procedure has enabled us to obtain esr spectra from samples subjected to a minimal amount of tissue disruption. Using samples obtained by this method we have assessed the effects of two other methods of tissue preparation (grinding and lyophilisation) and have also investigated to what extent ischaemia and reperfusion modify the susceptibility of the tissue to the artifactual production of radicals.

B. *Freeze-clamped Tissue*

The esr spectra of freeze-clamped aerobic, ischaemic and reperfused tissue were qualitatively similar; the most prominent peaks occurred at $g = 1.9$, $g = 2.005$, $g = 2.2$–2.6 and $g = 4.6$. Of particular interest was the isotropic peak seen at $g = 2.005$. This peak is characteristic of a semiquinone radical. Our observation of this peak in spectra from cardiac tissue is in agreement with other studies[29,30] although the changes that we observed in ischaemia (an increase in peak height and a decrease in peak width) differ from those seen by Nakazawa *et al.*[30] The narrowing of the signal width in our ischaemic samples was possibly due to the low concentration of oxygen in the tissue, a factor which would be expected to minimise line broadening.

C. *Freeze-clamped and Ground Tissue*

In addition to the iron signals that were observed in spectra from freeze-clamped tissue, an anisotropic signal ($g_{\parallel} = 2.040$ and $g_{\perp} = 2.008$), was observed in freeze-clamped, ground tissue from aerobic, ischaemic and reperfused heart. This signal is characteristic of an organic peroxyl radical.[20,28] In our study, the height of this signal was greatest in aerobic tissue, with a decrease to $33 \pm 10\%$ of this value in ischaemic tissue and to $76 \pm 26\%$ in reperfused tissue. Our observation of this peak in spectra of ground tissue is in agreement with recent reports[2,29] although the changes that we observed with ischaemia and reperfusion differ from those seen by others. Zweier *et al.*[17] report that the signal increased 2-fold in ischaemic tissue and 6-fold in reperfused tissue and Nakazawa *et al.*[30] report that ischaemic and reperfused tissue were the most susceptible to the generation of this anisotropic signal.

The complete absence of the anisotropic signal in heart tissue that had only been freeze-clamped, would suggest that the peroxyl radical signal seen in ground tissue is largely an artifact generated during tissue processing[20,28,29] possibly as a result of enzyme disruption incurred during the grinding process. The change of signal intensity with ischaemia and reperfusion, suggest that the susceptibility to radical generation alters with the oxidative state of the tissue.

D. *Freeze-clamped, Ground and Lyophilised Tissue*

In addition to the iron peaks observed in spectra from freeze-clamped tissue, the spectra of lyophilised tissue also exhibited an isotropic peak ($g = 2.009$). This peak is probably an artifact generated during lyophilisation.[18] Previous studies have indicated that the esr signals from lyophilised tissue are probably due to ascorbyl radicals, or other similar long-lived bio-radicals, which have been stabilised by adsorption onto the surface of a cellular component such as a protein.[31] An increase in the amount of cell damage prior to lyophilisation has been reported to promote

formation of this signal,[32] our results would agree with this hypothesis, since the signal height increased in both ischaemic (to 170 ± 57% of the aerobic value), and reperfused tissue (to 241 ± 85% of the aerobic value).

E. *Conclusions*

If artifactual generation of radicals is to be avoided, then the method of tissue preparation for esr spectroscopy is critical. Artifactual generation of radicals can occur as a consequence of tissue grinding or of lyophilisation. We have assigned the species that was generated by grinding of the tissue to an organic peroxyl radical. The identity of the signal generated by lyophilisation of the tissue remains unknown, although its intensity appears to correlate with the degree of cellular damage. Freeze-clamping using specially constructed aluminium tongs, to form frozen cylinders of tissue to fit directly into esr tubes, appears to be the least disruptive method for working with frozen tissue, as it eliminates the need for further tissue processing. Use of this technique indicates that a free radical species, assigned to a ubisemiquinone radical, is present in aerobic, ischaemic and reperfused rat heart tissue. Quantification of this signal, however, proved to be difficult; firstly, variations in the line width observed in spectra from samples collected during both ischaemia and reperfusion prevented the direct comparison of esr signal heights, and secondly, the underlying iron III signals prevented accurate measurement of signal areas. Unless these factors can be overcome, we would suggest that the direct examination of myocardial tissue using esr spectroscopy is quantitatively of little use in the investigation of reperfusion injury.

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References

1. McCord, J.M. Free radicals and myocardial ischemia: overview and outlook. *Free Rad. Biol. Med.*, **4**, 9–14 (1988).
2. Manning, A.S. Reperfusion-induced arrhythmias: do free radicals play a critical role? *Free Rad. Biol. Med.*, **4**, 305–316, (1988).
3. Hearse, D.J., Kusama, Y. and Bernier, M. Rapid development of singlet-oxygen-induced arrhythmias in the rat heart during photosensitisation of rose bengal. *J. Mol. Cell. Cardiol.*, **20**, (Suppl V), S.28, (1988).
4. Kusama, Y., Bernier, M. and Hearse, D.J. Singlet oxygen-induced arrhythmias: light response characteristics for photosensitisation of rose bengal. *J. Mol. Cell. Cardiol.*, **20**, (Suppl V), S.28, (1988).
5. Bernier, M., Hearse, D.J. and Manning, A.S. Reperfusion-induced arrhythmias and oxygen-derived free radicals. *Circ. Res.*, **58**, 331–340, (1986).
6. Hearse, D.J. Myocardial ischaemia and reperfusion: a possible role for free radicals? In: *Free radicals, oxidant stress and drug action* (ed. C. Rice-Evans). Richlieu Press, London, pp 13–42, (1987).
7. Hearse, D.J. and Tosaki, A. Free radicals and reperfusion-induced arrhythmias. Protection by PBN in the rat heart. *Circ. Res.*, **60**, 375–3983, (1987).
8. Hearse, D.J. and Tosaki, A. Reperfusion-induced arrhythmias and free radicals: studies in the rat heart with DMPO. *J. Cardiovasc. Pharmacol.*, **9**, 641–650, (1987).
9. Manning, A.S., Coltart, D.J. and Hearse, D.J. Ischaemia and reperfusion-induced arrhythmias in the rat. *J. Mol. Cell. Cardiol.*, **17**, 485–493, (1985).

10. Riva, E., Manning, A.S. and Hearse, D.J. SOD and the reduction of reperfusion-induced arrhythmias: in vivo dose-response studies in the rat. *Cardiovasc. Drugs Ther.*, **1**, 133–139, (1987).
11. Woodward, B. and Zakaria, M.V.M. Effect of some free radical scavengers on reperfusion-induced arrhythmias in the isolated rat heart. *J. Mol. Cell. Cardiol.*, **17**, 485–493, (1985).
12. Garlick, P.B., Davies, M.J., Hearse, D.J. and Slater, T.F. Direct detection of free radicals in the reperfused heart using ESR spectroscopy. *Circ. Res.*, **61**, 750–760, (1987).
13. Blasig, I.E., Ebert, B. and Lowe, H. Identification of free radicals trapped during myocardial ischaemia *in vitro* by esr. *Studia Biophysica.*, **116**, 35–42, (1986).
14. Arroyo, C.M., Kramer, J.H., Dickens, B.F. and Wegliki, W.B. Identification of free radicals in myocardial ischaemia/reperfusion by spin trapping with nitrone DMPO. *FEBS Lett.*, **221**, 101–104, (1987).
15. Zweier, J.L. Measurement of superoxide-derived free radicals in the reperfused heart. *J. Biol. Chem.*, **263**, 1353–1357, (1988).
16. Bolli, R., Patel, B.S., Jeroudi, M.O., Lai, E.K. and McCay, P.B. Demonstration of free radical generation in "stunned" myocardium of intact dogs with the spin trap α-PBN. *J. Clin. Invest.*, **82**, 476–485, (1988).
17. Zweier, J.L., Flaherty, J.T. and Weisfeldt, M.L. Direct measurement of free radical generation following reperfusion of the ischaemic myocardium. *Proc. Nat. Acad. Sci. USA*, **84**, 1404–1407, (1987).
18. Heckly, R.J. and Dimmick, R.L. Electron paramagnetic resonance in frozen and dried biological materials. *Nature*, **216**, 1003–1004, (1967).
19. Heckly, R.J. Free radicals in dry tissues. In: *Biological Applications of Electron Spin Resonance* (eds H.M. Swartz, J.R. Bolton, D.C. Borg). Wiley-Interscience, New York, pp 197–212, (1972).
20. Benedetto, C., Bocci, A., Dianzani, M.U., Ghiringhello, B., Slater, T.F., Tomasi, A. and Vannini, V. ESR studies on normal uterus and cervix and on benign and malignant uterine tumors. *Cancer Res.*, **41**, 2936–2942, (1981).
21. Urbanski, T. Formation of free radicals by mechanical action. *Nature*, **216**, 577–578, (1967).
22. Chandra, H. and Symons, M.C.R. Sulphur radicals formed by cutting α-keratin. *Nature*, **328**, 833–834, (1987).
23. Langendorff, O. Untersuchungen am uber lebenden Saugertierherzen. *Pflugers Archives*, **61**, 291–332, (1895).
24. Palmer, G. EPR of metalloproteins. *Biochem. Soc. Trans.*, **13**, 548–560, (1985).
25. Swartz, H.M. Cells and tissues. In: *Biological Appl. Electron Spin Reson.* (eds H.M. Swartz, J.R. Bolton, D.C. Borg). Wiley-Interscience, New York, pp 155–197, (1972).
26. Davies, K.J.A. and Hochstein, P. Ubisemiquinone radicals in liver: implications for a mitochondrial Q cycle in vivo. *Biochem. Biophys. Res. Commun.*, **107**, 1292–1299, (1982).
27. Suzuki, H. anbd King, T.E.J. Evidence of an ubisemiquinone radical(s) from the NADH-ubiquinone reductase of the mitochondrial respiratory chain. *J. Biol. Chem.*, **258**, 352–358, (1983).
28. Tomasi, A., Benedetto, C. and Slater, T.F. Identification of a strong free radical signal that is detectable in uterine cervix. In: *Cancer of the Uterine Cervix. Biochemical and Clinical Aspects* (eds D.C.H. McBrien and T.F. Slater), Academic Press, Orlando, pp 265–275, (1984).
29. Baker, J.E., Felix, C.C., Olinger, G.N. and Kalyanaraman, B. Myocardial ischaemia and reperfusion: direct evidence for free radical generation by esr. *Proc. Nat. Acad. Sci. USA*, **85**, 2786–2789, (1988).
30. Nakazawa, H., Ichimori, K., Okino, H. and Hori, S. Is O_2^- demonstration by electron-spin resonance spectroscopy really O_2^-? *Am. J. Physiol.*, **255**, H213–H215, (1988).
31. Swartz, H.M. and Dodd, N.J.F. Ascorbate and radical reactions in vivo. In: *Oxygen and Oxy-Radicals in Chemistry and Biology* (eds M.A.J. Rodgers, E.L. Powers). Academic Press, New York, pp 161–168, (1981).
32. Dodd, N.J.F. and Swartz, H.M. ESR and lyophilised tissue and its relevance to malignancy. *B. J. Cancer*, **49**, 65–71, (1984).

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