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Hydroxyl Radical and Its Detection Methods

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Abbreviation:
CE: capillary electrophoresis
DHBA: dihydroxybenzoic acid
DMPO: 5,5-dimethylpyrroline-*N*-oxide
EPR: electron paramagnetic resonance
ESI: electrospray ionization
ESR: electron spin resonance
GC–MS: gas chromatography–mass spectrometry
HBA: hydroxybenzoic acid
HPLC–ED: high-performance liquid chromatography electrochemical detection
PBN: phenyl -N- *tert*-butylnitrone
ROS:reactive oxygen species
TBDMS: *tert.*-butyldimethylsilyl

Abstract

Under physiological and pathological conditions, the reactive oxygen species (ROS) are formed from both exogenous and endogenous sources. ROS are implicated in a wide number of disease processes, including atherosclerosis, autoimmune disorders, rheumatoid arthritis, neuronal degeneration, cardiovascular diseases and cancer. Among the reactive oxygen species produced in living cells, hydroxyl radical is the most active and strongest oxidant agent, and can react with almost any substance at its diffusion rate. When hydroxyl radicals are generated in excess or the cellular antioxidant defense is deficient, they can stimulate free radical chain reactions by interacting with proteins, lipids and nucleic acids, causing cellular damage. Therefore, confident analytical approaches are needed to ascertain the importance of hydroxyl radicals in biological systems. Until now, several effective methods have been established.

Keywords**:** Hydroxyl radicals, detection

The study of the influence of free radicals in the biological process depends primarily on the capacity to detect these reactive species.

1. Introduction

Reactive oxygen species (ROS), such as superoxide and hydroxyl radicals are formed continuously in the body as a result of various biochemical processes [1,2]. They play important roles in killing both bacteria and tumor cells as well as in signal transduction [1]. However, overproduction of ROS in the body inversely causes tissue damage [1,2]. Of all the ROS, the hydroxyl radical is the most reactive oxygen radical *in vivo* [3,4,5]. In general, the hydroxyl radical is generally considered as a harmful byproduct of oxidative metabolism, causing molecular damage in living systems. It is also implicated in various processes such as mutagenesis, aging, and a series of pathological events. It is well established that levels of free iron play a critical role in initiating and catalyzing a variety of radical reactions in the presence of oxygen [5]. Copper is also important in hydroxyl radical genesis [6]. Living systems have a complex antioxidant defense system, including the antioxidant enzymes and non-enzymatic small molecular antioxidants [2,5]. Generally, cellular levels of antioxidant enzymes and non-enzymatic antioxidants respond to levels of oxygen and ROS. This mechanism enables cells to defend against oxyradical production [2].

2. Hydroxyl radical generation

The hydroxyl radical is an oxygen-containing chemical species that has the capacity to abstract an electron from a variety of organic or inorganic molecules and atoms. Through the metal (M) ion-depend breakdown of hydrogen peroxide, transition metals, especially free ion and copper has a central role in the production of OH • *in vivo.*

$O_2.^- + M^{n+} \rightarrow M^{(n-1)+}$ (Eq. 1) M=Cu (n=2) or Fe (n=3)

$M^{(n-1)+} + H_2O_2 \rightarrow M^{n+} + OH^- + OH\cdot$ (Eq. 2)

Specially, if the metal is ferrous ions, Eq. 2 is called Fenton's reaction:

$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH\cdot$ (Eq. 2. 1)

Besides the generation of OH• through the Fenton reaction, The other pro-oxidant effects of iron have also been demonstrated *in vivo*, including the stimulation of membrane lipid peroxidation, the formation of hypervalent oxidizing complexes, the promotion of oxidative DNA damage, and the sensitization of cells to various oxidants [9]. Because of the toxicity o free ion and copper, they are tightly controlled in organisms. In organism, iron is predominantly bound transferring. Various biological iron chelates have been assayed for efficiency of Fenton catalysis (percentage conversion of O_2^- to .OH) and ranked: citrate >> pyrophosphate > lactate > adenosine triphosphate > hematin, transferrin, hemin, and hemoglobin [9]. Also, copper has been suggested to facilitate oxidative tissue injury through a free radical-mediated pathway analogous to the Fenton reaction [6].

The second way of forming hydroxyl radical *in vivo i*s the soluble copper or iron catalyzed Haber–Weiss reaction [2,6]:

$H_2O_2 + O_2^-\cdot \rightarrow O_2 + OH^- + OH\cdot$ (Eq. 3)

The third way of forming hydroxyl radical *in vivo i*s through interaction between the superoxide radical and nitric oxide [5,7]:

$O^-_2\cdot + NO\cdot \rightarrow ONOO^- + H^+ \rightarrow ONOOH \rightarrow OH\cdot + NO_2\cdot \rightarrow NO^-_3 + H^+$ (Eq. 4)

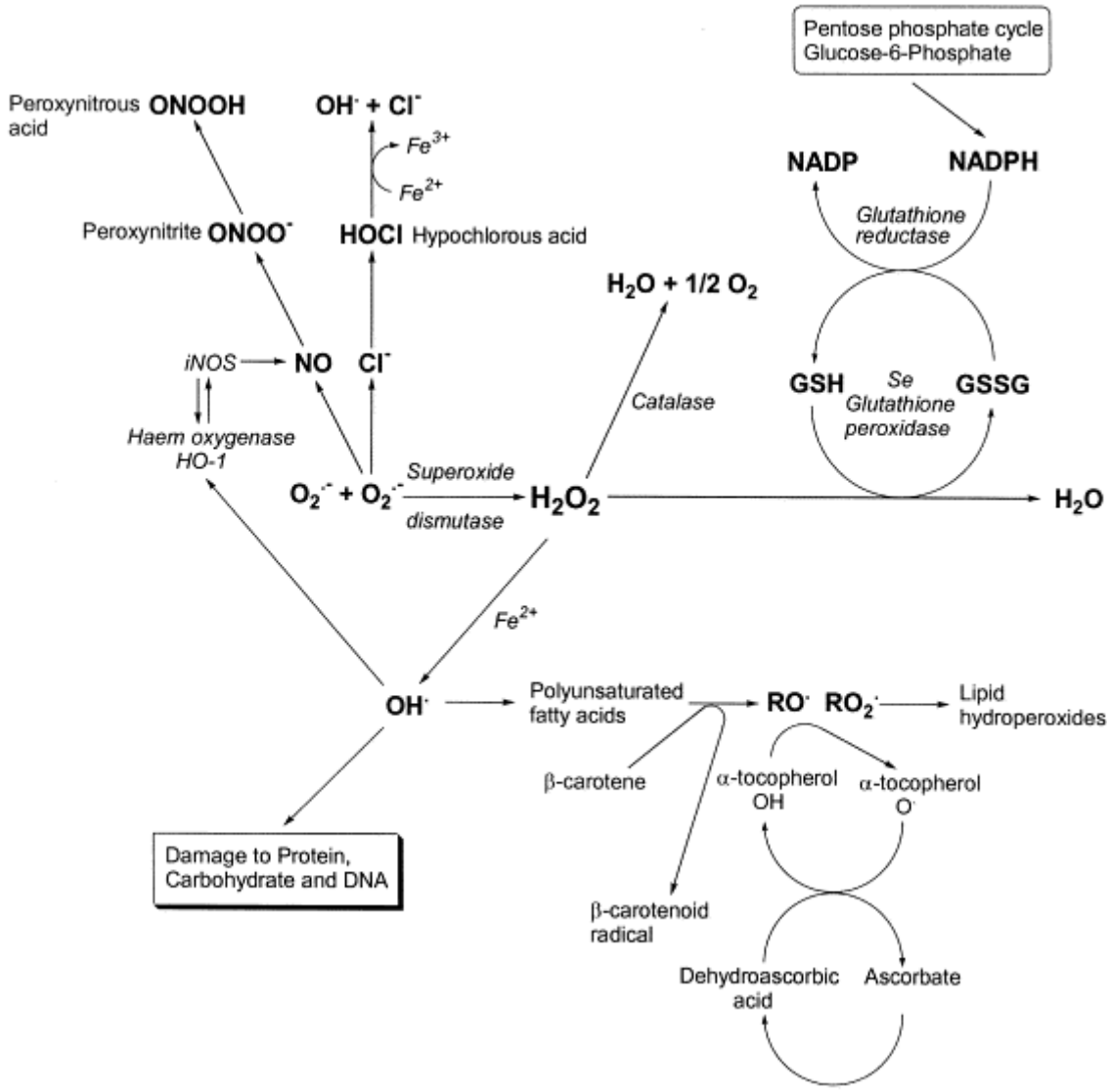


Fig. 1: The main pathway of production of OH • *in vivo* and some important molecular related to OH • Ref. [5].

Haem oxygenase 1 (HO-1) is an inducible enzyme that catalyses the rate limiting step in the degradation of haem to biliverdin, carbon monoxide and iron. Activation of HO-1 is an ubiquitous cellular response to oxidative stress. Recent studies have suggested an important role for HO-1 in the control of iNOS. HO may participate in a coupled cellular protection mechanism in which the iron-storage protein, ferritin, ultimately provides the protection by sequestering and oxidizing the iron released by the HO-catalyzed breakdown of heme. HO activity influences the

de novo synthesis of ferritin apoprotein. Before its sequestration by ferritin, the released iron may be available for the catalysis of deleterious oxidation reactions.So the induction of HO activity may have both pro- and antioxidant sequelae depending on cellular redox potential, and the metabolic fate of the heme iron [9,10].

3. Analysis of hydroxyl radical

Many techniques have been used to detect hydroxyl radicals *in vitro* and *in vivo* [2,5,6]. The methods for the determination of hydroxyl radical include the ESR system, which measures the ESR spectrum of a spin adduct derivative after spin trapping [6], or chromatographic methods, which determine the trapping products after they have been reacted with hydroxyl radicals.

3.1. High-performance liquid chromatography

Because the ESR methods require a sophisticated and high-cost instrument system, and spin-trapped free radicals are still extremely short-lived, HPLC techniques have been developed to separate and quantify the stable hydroxylated derivatives in biological systems [5].

In the use of HPLC systems, salicylate is included in the perfusion buffer at concentrations ranging from 0.1 to 2 m*M*, depending on the detection apparatus employed. There is a much higher efficiency (approximately 100%) in a coulometric detector when compared to a standard amperometric detector (approximately 1–5%) [2].

3.1.1. Electrochemical detection (ED)

HPLC–ED is one of the most important methods for the *in vivo* measurement of the hydroxyl radical, and is based on the ability of the hydroxyl radical to attack the benzene rings of aromatic molecules. The formation of 8-hydroxy-deoxyguanosine (8-OH-dG), of which the hydroxyl radical reacts with DNA or 2-deoxyguanosine can be effectively separated and detected from deoxyguanosine by HPLC–ED. The sensitivity of electrochemical detection is about 1000 times better than optical detection, ESR or HPLC with ultraviolet detection [2].

Although HPLC–ED is a very sensitive method for trapping hydroxyl radicals using salicylic acid or 4-HBA, a small amount of hydroxylated contaminants (2,3-DHBA, 2,5-DHBA and 3,4-DHBA) will be traced during the determination of hydroxylation products of the various substrates. This was confirmed by their identification using GC–MS with *tert.*-butyldimethylsilyl (TBDMS) derivative in the microdialysis sampling technique. Plausible factors affecting hydroxylation of DHBAs using microdialysis technique include time, use of metal ions, and reuse of microdialysis probes. The longer the infusion contained 4-HBA, the more 3,4-DHBA was produced per given time period [2].

3.1.2. Mass spectrometry (MS)

A mass spectrum is obtained by converting sample species into rapidly moving gaseous ions and separating them based on their mass-to-charge ratios. So far, MS is considered to be the most widely applicable analytical tool available, as it is capable of providing information on the structure of molecular species and the isotope ratios of atoms in sample species. In addition to combination with GC, MS has been coupled with liquid chromatography for the analysis of samples that contain non-volatile constituents. Therefore, MS combined with HPLC has become

the most powerful tool for the identification of molecular species in biological samples. Domingurs et. al. demonstrated that mass spectrometry can be a powerful tool in the detection and identification of spin adducts of DMPO with hydroxyl radical species. In addition, Mass spectrometry can be used in detection of free hydroxyl radicals in complex mixtures, since mass spectrometry is able to discriminate these adducts in such situations [7].

3.2 Gas chromatography (GC)

GC is applied in the analysis of volatile and thermally stable constituents in samples. But most of the bio-species related to hydroxyl radicals in living systems are hydrophilic and thus have low volatility, the derivatization of species with an appropriate reagent is carried out before GC determination. Most derivatization reactions are methylation with diazomethane and silylation with bis(trimethsilyl)trifluoroacetamide, etc. to increase hydrophobicity and volatility of analytes. Usually the hydroxyl radical is ion-trapped by adding a reagent, or by the species existing in cells. After appropriate pretreatment processes, such as extraction and derivatization, the adduct-species is injected on to the GC system for separation and detection [2].

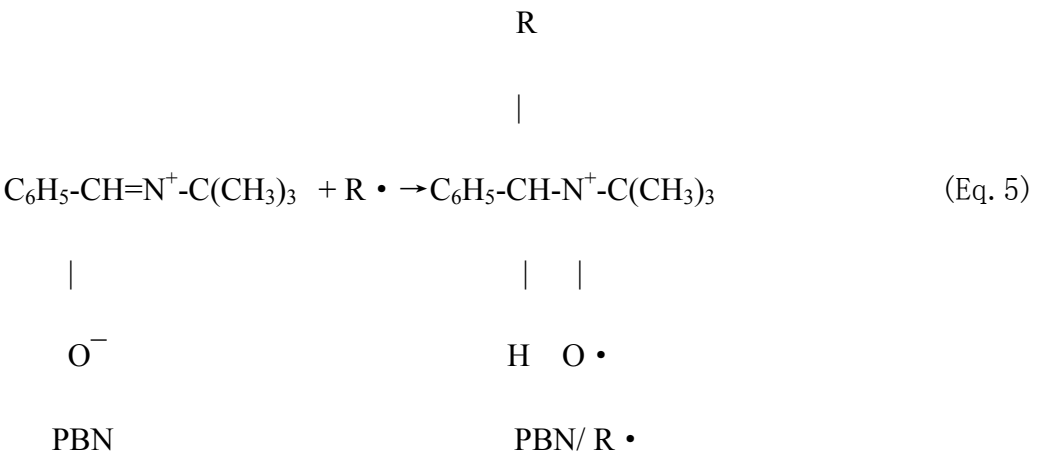
3.3 Capillary electrophoresis (CE)

The application of CE for monitoring of oxidative damage caused by free radicals might be limited by its difficult detection. However, with the improvements of detection and combined techniques, such as laser-induced fluorescence detection, interfaced on-line to a mass spectrometer (MALDI–TOF, matrix-assisted laser desorption ionization time-of-flight, MS–MS, and ESI-TOF-MS, etc.), CE will become a successful analytical tool with widespread applications in the biological sciences [2].

3.4 Electron spin resonance (ESR)

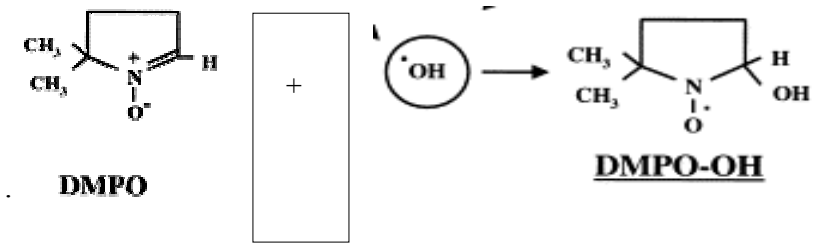
3.4.1Basic spin trapping technique

Endogenous free radicals produced in living systems have extremely short half-lives and in low concentrations. So spin trapping technique has been developed to overcome these difficulties, in which a nitrone or nitroso compound is allowed to react with a free radical to produce a nitroxide whose stability is considerably greater than that of the parent free radical [1,7]. Among the several nitrones used as spin traps, -phenyl -N- *tert*-butylnitrone (PBN) and 5-dimethyl-1-pyrroline *N*-oxide (DMPO) are commonly employed [1].



(Eq. 5)

PBN is a stable compound and forms relatively long-lived spin adducts with various types of radicals. Kadiiska et al.demonstrated *in vivo* hydroxyl radical generation in the bile of rats 10 weeks after the rats were fed an iron-loading diet and 40 min after the rats were injected via the intraperitoneal route with PBN dissolved in dimethyl sulfoxide (DMSO). Hydroxyl radicals generated *in vivo* are converted to methyl radicals via reaction with DMSO, the methyl radical is then detected as its adduct with PBN by ESR spectroscopy [6,11].



(Eq. 6)

Figure 2: The ESR spectrum of DMPO-OH adduct.

DMPO is water-soluble, and its reaction rate constant for superoxide and hydroxyl radicals is high. Moreover, spin trapping analysis using DMPO makes it possible to distinguish simultaneously among a variety of important biologically generated free radicals [1]. If DMSO is added to a hydroxyl radical–generating system, the resulting reaction produces methyl radical, which can react with DMPO to yield DMPO-CH_3 with its characteristic hyperfine splitting pattern. Verification of hydroxyl radical formation can be accomplished by the addition of DMSO [1,2].

3.4.2 In vivo detection of oxidative stress by ESR

Owing to limitations of ESR sensitivity, only those free radical species with relatively long half-lives are measurable. With the development of spin trapping methods, it has become possible to detect short-lived free radical species using conventional X-band ESR instrumentation. Recently, ESR instruments operating at low frequencies (L-band ESR at <1

GHz) have made it possible to measure radical species in the whole animal [8]. Therefore, it is expected that L-band ESR can be adapted to measure oxidative stress *in vivo*. If the spatial distribution of free radicals generated in organs could be visualized in living animals, it would provide information on the pathophysiological role of free radicals in the body. Because only a limited number of free radicals are generated and they are short lived, it is difficult to obtain their direct images in the body. Halpern et al first measured the production of free radicals in the tissue of a living animal using a low-frequency 260 MHz spectrometer and -(4-pyridyl-1-oxide)-N-*tert*-butylnitrone (4-POBN) plus ethanol. They detected an ESR spectrum characteristic of 4-POBN-CH(OH)-CH_3 originating from hydroxyl radicals produced during radiation in a leg tumor (12 mm diameter) in living mice [12].

4. Conclusion

This review mainly states the chemistry of hydroxyl radical formation *in vivo* and its detection methods. Among the main pathways of hydroxyl radical formation, Fenton reaction has a predominant status. As the most active free radical *in vivo*, hydroxyl radical can react with almost any substance at its diffusion rate and is extremely toxic to organism. So it is under strict controlled by complex antioxidant defense system, including the antioxidant enzymes and non-enzymatic small molecular antioxidants in life systems. The study of the influence of hydroxyl radical in the biological process depends primarily on the capacity to detect hydroxyl radical. Until now, several analytical techniques applied in the measurement of the hydroxyl radical, such as HPLC–ED, HPLC–MS, GC–MS, CE, ESR. Among these methods, spin trapping technique in ESR is most widely used. And the recent improvements make the in vivo detection of hydroxyl radical possible.

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