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ELECTRONIC SPIN RESONANCE DETECTION OF HYDROXYL RADICALS AND SINGLET OXYGEN DURING PEROXIDATION OF BOPINDOLOL IN THE PRESENCE OF COPPER IONS

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New evidence for generation of singlet oxygen ($^1O_2$) and hydroxyl radical ($HO^\bullet$) during peroxidation of bopindolol in the presence of Cu ions has been found using electron spin resonance (ESR) and spectrophotometry methods. 2,2,6,6-tetramethyl-4-piperidone and 5,5-dimethyl-1-pyrroline-1-oxide were used as traps. The spectrophotometry determination of $^1O_2$ was based on bleaching of *p*-nitrosodimethylaniline (RNO), which was caused by the product of the reaction of $^1O_2$ with imidazole. The effect of $^1O_2$ quenchers and oxygen free radical scavengers on ESR signal and on bleaching of RNO has been studied. The results demonstrate the enhancing effect of bopindolol on the generation of $HO^\bullet$ and $^1O_2$ in the Fenton reaction.

INTRODUCTION

Reactive oxygen species (ROS) such as superoxide radical anion ($O_2^{\bullet-}$), hydroxyl radical ($HO^\bullet$), hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) are formed in normal metabolic processes (Bartosz, 2003). Environmental pollution, infections and certain drugs may be another source of ROS and an additional burden for organism. The diseases are related to the imbalance between of ROS formation and their elimination by antioxidants (Halliwell & Gutteridge, 1989). All ROS have the ability to interact with cellular compounds such as proteins, polyunsaturated lipids, carbohydrates and nucleic acids (Kruk, 2008; Bartosz, 2003). Radical related damage of DNA and protein have been proposed to play a key role in the development of various serious diseases such as cancer, atherosclerosis, neoplasm, diabetes, neurodegenerative disturbances (Halliwell, 1989; Droge, 2002; Kehrer, 1993). Electron spin resonance (ESR) is spectroscopic technique which allows to detect free radicals even in complex chemical and biological systems (Finkelsen, Rosen & Rauckmam, 1980; Khan & Swartz, 2002; Bartosz, 2006; Bacic', Spasojevic', Secerov & Mojovic', 2008). As oxygen species are short-lived, and in most cases the sensitivity of ESR spectroscopy is insufficient for their detection, stabilizer molecules called spin traps are used for their detection. Spin traps react with these short-lived radicals, producing long-lived radical adducts. For this reason the spin-trapping technique is uniquely important method investigation of generation of oxygen species.

Bopindolol (Fig.1) is a β-adrenergic blocker which is clinically used in the treatment of hypertension and cardiac arrhythmias. We previously reported that the peroxidation of bopindolol catalyzed by Co(II)-EDTA is accompanied by chemiluminescence (Aboul-Enein, Kruk & Lichszteld, 1998; Kruk, Michalska, Kładna & Aboul-Enein, 2002). The results showed that oxygen species ($O_2^{\bullet-}$, $HO^\bullet$) were precursors of $^1O_2$ being emitters of light emission. In the present analyses we followed up these results dealing with peroxidation of bopindolol using Cu ions as a catalyst and the spin traps 2,2,6,6-tetramethyl-4-piperidone and 5,5-dimethyl-1-pyrroline-1-oxide for reaction with $^1O_2$ and HO radicals, respectively.

MATERIALS AND METHODS

Bopindolol was kindly provided by Sandoz (Basel, Switzerland). The compounds that were used as antioxidants were obtained from Merck (Darmstadt, Germany). 2,2,6,6-tetramethyl-4-piperidone-N-oxide (TEMPO), 2,2,6,6-tetramethyl-4-piperidone (TEMP), 5,5-dimethyl-1-pyrroline-1-oxide (DMPO), imidazole (A) and *p*-nitrosodimethylaniline (RNO) were purchased from Sigma (St. Louis MO). Other reagents were analytical grade from POCH (Gliwice, Poland). DMPO was prepared as a 1M aqueous solution immediately

before use and stored in darkness at -15°C to avoid any

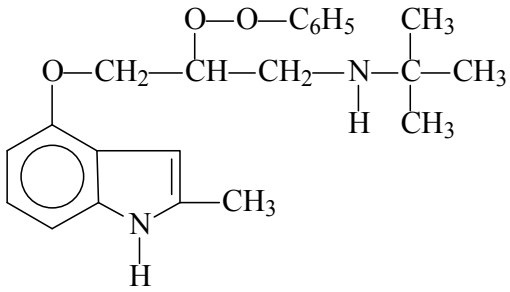

Fig. 1. The chemical structure of bopindolol.

oxidation of the spin trap. When TEMP was used as a spin trap, the spin-trapping experiments were performed using solutions prepared by dissolving an appropriate amount of reagents in methanol. The pH of the solutions was increased by the addition of a few drops of 0.1M NaOH (dissolved in methanol), yielding a final pH of approximately 9.2.

The ESR measurements utilized Bruker E500 spectrometer (X-band) operating at 9.77 GHz with modulation frequency 100 kHz. The flat quartz cell (0.25-mm internal thickness) containing the reaction mixture was placed in cavity of the apparatus. The spectra were recorded with nonsaturating microwave power level of 0.63 mW and a modulation amplitude of 1 G.

The spectrophotometric determination of $^1O_2$ was performed according to the method of Kraljic' and Moshni (Kraljic' & Moshni, 1978). The method is based on the bleaching of RNO that is caused by an intermediate product of the reaction of $^1O_2$ with imidazole (A) which is transannular peroxide (AO_2).

$$^1O_2 + A \rightarrow AO_2 \quad (1)$$
$$AO_2 + RNO \rightarrow -RNO + \text{products}$$

The bleaching of RNO was followed by monitoring the decrease in the optical density at 440 nm. A Zeiss M-40 spectrophotometer was used for the spectrophotometric measurements. All experiments were done in triplicate. The data are presented as the mean standard with deviation.

RESULTS AND DISCUSSION

It has been shown that the TEMP is the specific $^1O_2$ spin trap since neither $O_2^{\bullet-}$ nor $HO^\bullet$ and H_2O_2 produce the nitroxide radical (Zang, van Kuijk, Mira & Mira, 1995). The spin trap reacts with $^1O_2$ to form a relatively long-lived TEMP-$^1O_2$ adduct (TEMPO). The ESR spectrum of TEMPO consists of a triplet line (intensity 1:1:1) with a hyperfine splitting constant of 15.9 ± 0.2 G and a line width of 0.40 ± 0.02 G (g value of 2.0062). A typical ESR spectrum of the spin adduct of singlet oxygen measured from the TEMP + Cu(II) + bopindolol system is presented in Fig.2. The spectrum parameters: hyperfine splitting constant (16.0 ± 0.02 G), linewidth (0.38 ± 0.02 G) and splitting factor (2.0065) are almost the same and as that measured by us for commercial TEMPO under the same condition (data not shown) and essentially identical with that from the literature data. The kinetic of the build-up in the TEMPO radical ESR signal is shown in Fig.2 (curve 1), in which the rate of $^1O_2$ formation is proportional to the duration of bopindolol peroxidation. The intensity of the signal increased reading the maximum within about 15 min. When bopindolol was missing (curve 2), the intensity of ESR signal was decreased approximately 2 times. Typical $^1O_2$ quencher, β-carotene added to the reaction mixture reduced the ESR signal intensity (curve 3). β-carotene is known to be a powerful antioxidant in the hydrophobic phase of a cell (Bellus', 1979) although it exhibits also scavenging ability toward the HO radical. β-carotene could be applied as a scavenger of $^1O_2$ because we used methanol as a solvent, the reagent known with a high rate constant (10^9 $M^{-1}s^{-1}$) in reaction with $HO^\bullet$ (Neta & Dorfman, 1968). The choice of methanol as the solvent is also important because of about four times longer life time of $^1O_2$ in methanol than in H_2O. The ESR signal was hardly detectable when H_2O_2 was omitted.

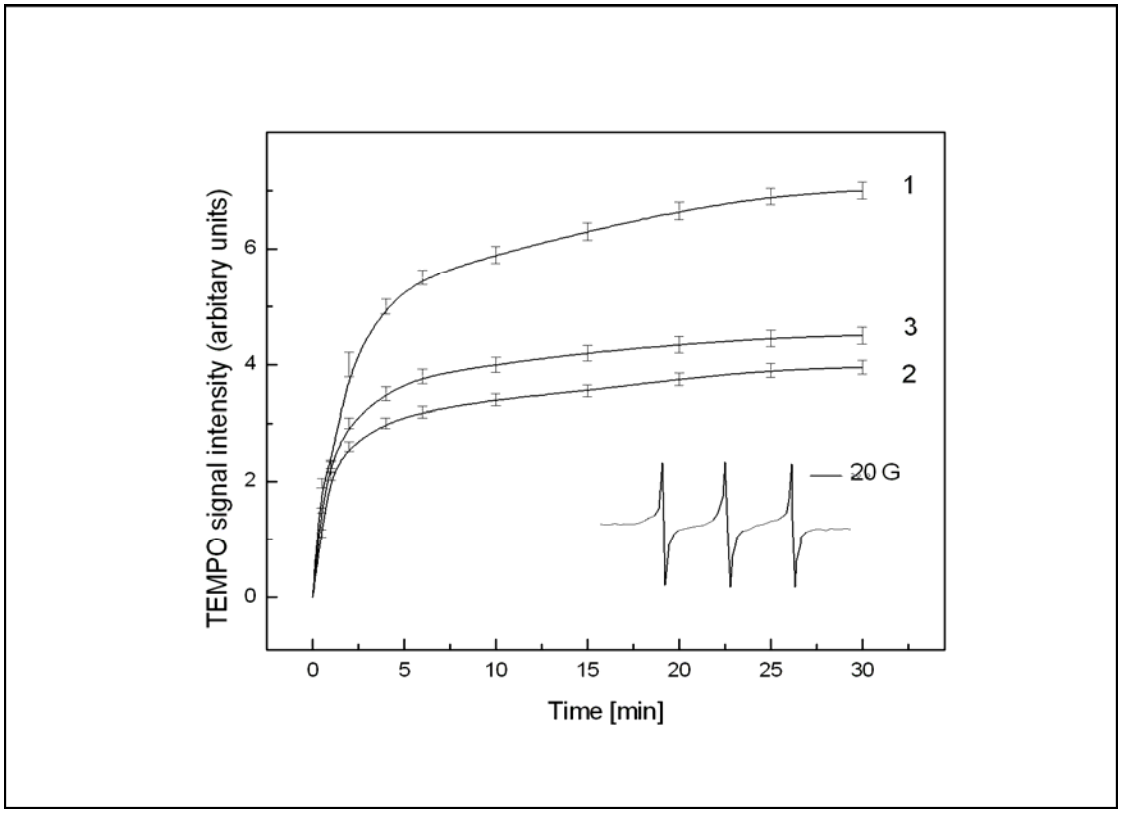

Fig. 2. The dependence TEMPO radical signal intensity on the peroxidation time of 0.5 mM bopindolol in the presence of 0.5 mM H_2O_2 and 0.5 mM $CuSO_4$ (curve 1). Curve 2- the same conditions as curve 1 except that bopindolol was omitted. Curve 3- the same as curve 1 except that 0.02 mM β-carotene was added. The concentration of TEMP was 0.25 mM in 0.1 M CH_3ONa. Temperature 297 K. Insert: the ESR signal TEMPO under the same reaction conditions as curve 1.

The obtained results strongly suggest that $^1O_2$ is generated during oxidation of bopindolol by the Fenton reagent ($CuSO_4$ + H_2O_2) as well during decomposition of H_2O_2 in the presence of Cu ions.

As reported previously , the reaction between metal ions, bopindolol and H_2O_2 produces HO radical and $^1O_2$ as follows (Aboul-Enein, *at al.*,1999, Kruk *at al*., 2002):

$$Cu(I) + H_2O_2 \rightarrow Cu(II) + HO^{\bullet} + HO^-$$

(Fenton- like reaction)

$$Cu(I) + O_2 \rightarrow Cu(II) + O_2^{\bullet -}$$

$$H_2O_2 + HO^- \rightarrow HOO^- + H_2O$$

$$H_2O_2 + HOO^- \rightarrow HO^- + H_2O + {}^1O_2$$

$$O_2^{\bullet -} + H_2O_2 \rightarrow HO^{\bullet} + HO^- + {}^1O_2$$

$$O_2^{\bullet -} + O_2^{\bullet -} + 2H^+ \rightarrow H_2O_2 + {}^1O_2$$

$$O_2^{\bullet -} + HO^{\bullet} \rightarrow HO^- + {}^1O_2$$

To investigate the formation of HO radical, which is recognized as a precursor of $^1O_2$, we used DMPO as a spin-trap. The spin-trap DMPO was successfully applied to trap HO radicals and $O_2^{\bullet -}$ by several researchers (Finkelstein *at al*., 1980; Buettner, 1993; Bergman, Perelman, Dubinsky & Grossman, 2003). The DMPO-$HO^{\bullet}$ adduct shows an ESR spectrum consisting of a quartet line with an intensity ratio of 1:2:2:1 ($A_N = A_H$ = 14.9 G). The spectrum is rather stabile, whereas the DMPO-$O_2^{\bullet -}$ adduct is highly unstable and spontaneously decays into the long-lived DMPO-$HO^{\bullet}$ adduct radical (the half-live 2.6 h) (Finkelstein *at al.*, 1980). The half-live of the DMPO-OOH adduct ranged from 27 s at pH 9 to 91 s at pH 5 (Buettner & Oberley, 1978). The ESR spectrum of spin trap DMPO formed during peroxidation of bopindolol is shown in Figure 3(a).

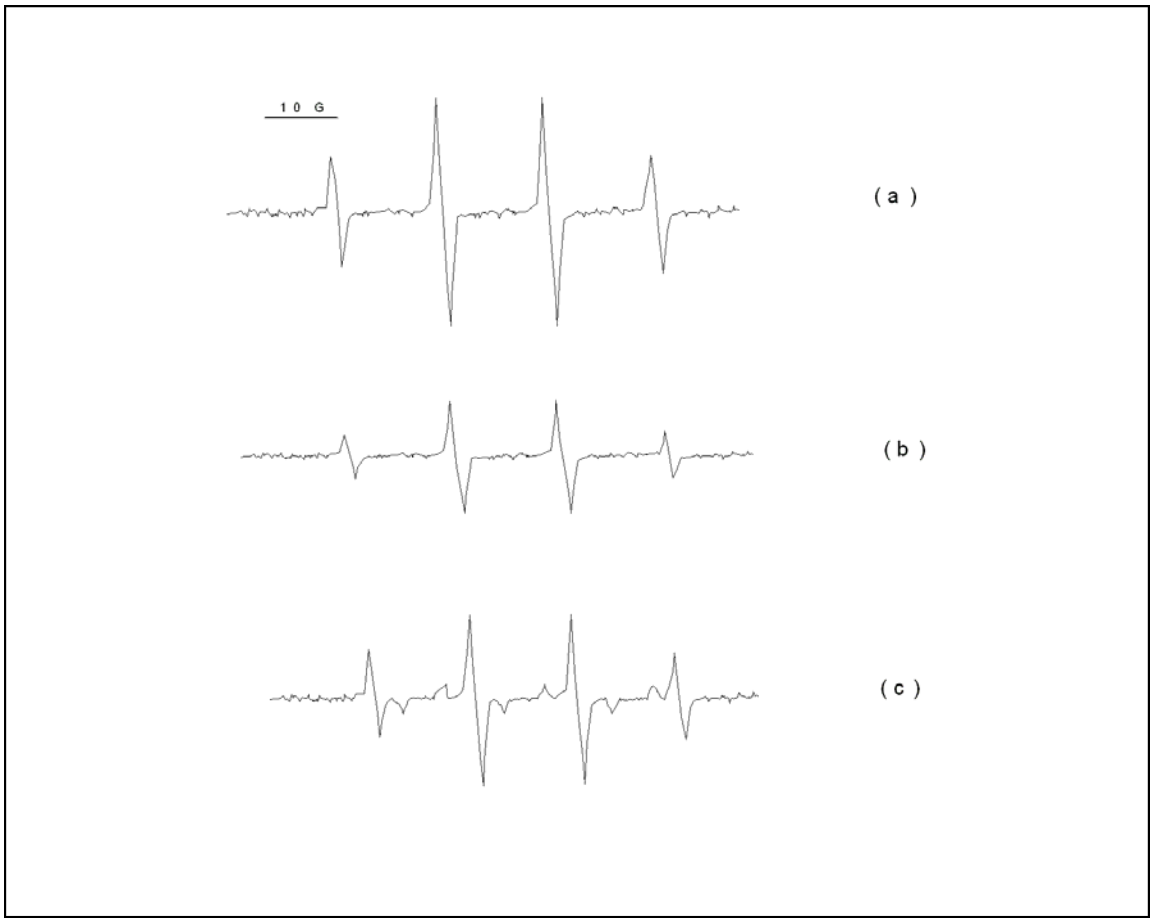

Fig. 3. (a) ESR spectra of DMPO-bopindolol-$CuSO_4$- H_2O_2 detected 15 min after mixing of reagents; (b)- the same as (a) but in the absence of bopindolol; (c)- the same as (a) but in the presence of ethanol (15% v/v). Concentration: 50 mM DMPO, 0.5 mM bopindolol, 0.5 mM $CuSO_4$, 0.5 mM H_2O_2, carbonate buffer 0.1 M, pH 9.2. Temperature 296 K.

The observed spectrum registered 15 min after mixing of reagents was characterized by hyperfine coupling constants of $A_N = A_H = 14.88 \pm 0.04$ G and g = 2.0060. This suggests that the observed radical is the DMPO-$HO^{\bullet}$ adduct. Because the detection of DMPO-$HO^{\bullet}$ does not necessarily mean that HO radical has been trapped, we verified that result using ethanol. The addition of ethanol should inhibit also the DMPO-$HO^{\bullet}$ signal and result using in the appearance of a new signal due to trapping of the α–hydroxyethyl radical formed in the reaction of $HO^{\bullet}$ with ethanol (Finkelstein et al., 1980). Indeed, we observed the appearance of the α–hydroxyethyl radical ($CH(OH)CH_2$) spectrum that is six-line and decrease of the DMPO-$HO^{\bullet}$ signal (data not shown). Figure 3(b) presents the DMPO-$HO^{\bullet}$ adduct formed in the absence of bopindolol, and part(c) the significant inhibition of the spin adduct formation in the presence of ethanol. These findings indicate that bopindolol enhances generation of HO radicals and that detection of the corresponding DMPO-$HO^{\bullet}$ is a good indicator for $HO^{\bullet}$ formation under our experimental conditions. This observation also suggests that the DMPO-$HO^{\bullet}$ adduct originates from trapping of $HO^{\bullet}$ and not from the decomposition of DMPO-$O_2^{\bullet-}$ adduct. The time profiles of the DMPO-$HO^{\bullet}$ adduct formation during peroxidation of bopindolol are shown in Fig. 4. The intensity of DMPO-$HO^{\bullet}$ signal detected from the $CuSO_4$-H_2O_2 system (curve 2) was greatly increased in the presence of bopindolol (curve 1). The presence of ethanol in the bopindolol + $CuSO_4$ + H_2O_2 system (curve 3) resulted in the decrease of the ESR signal.

The second method that we used to detect the generation of $^1O_2$ during oxidation of bopindolol is the bleaching of RNO (Fig. 5). In the absence of imidazole the loss of RNO was not measurable.

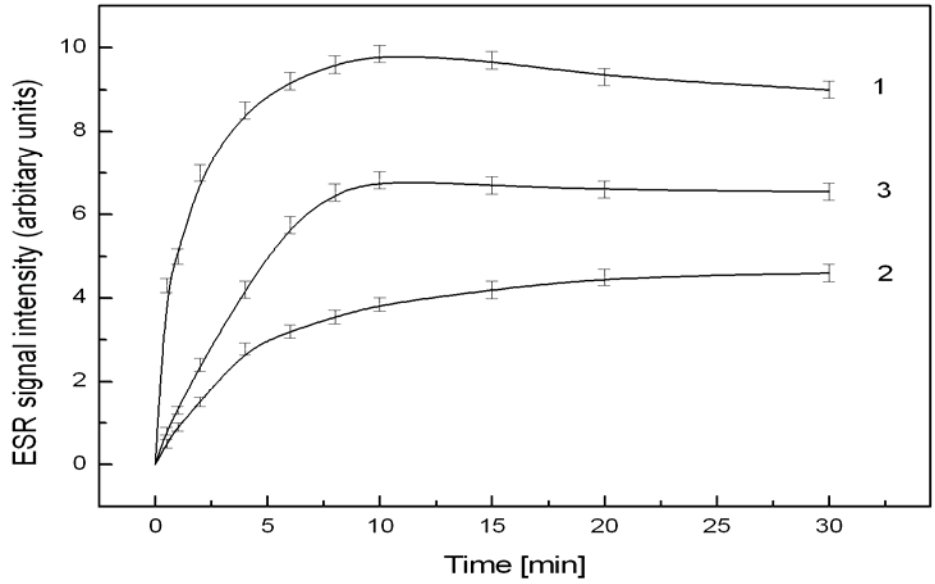

Fig. 4. The dependence of intensity of DMPO signal on the peroxidation time of 0.5 mM bopindolol in presence of 0.5 mM H_2O_2 and 0.5 mM $CuSO_4$ (curve 1). Curve 2- the same as curve 1 except that bopindolol was omitted. Curve 3- the same as curve 1 but in the presence of ethanol (15% v/v). The concentration of DMPO was 50 mM, 0.1M carbonate buffer pH 9.2. Temperature 296 K.

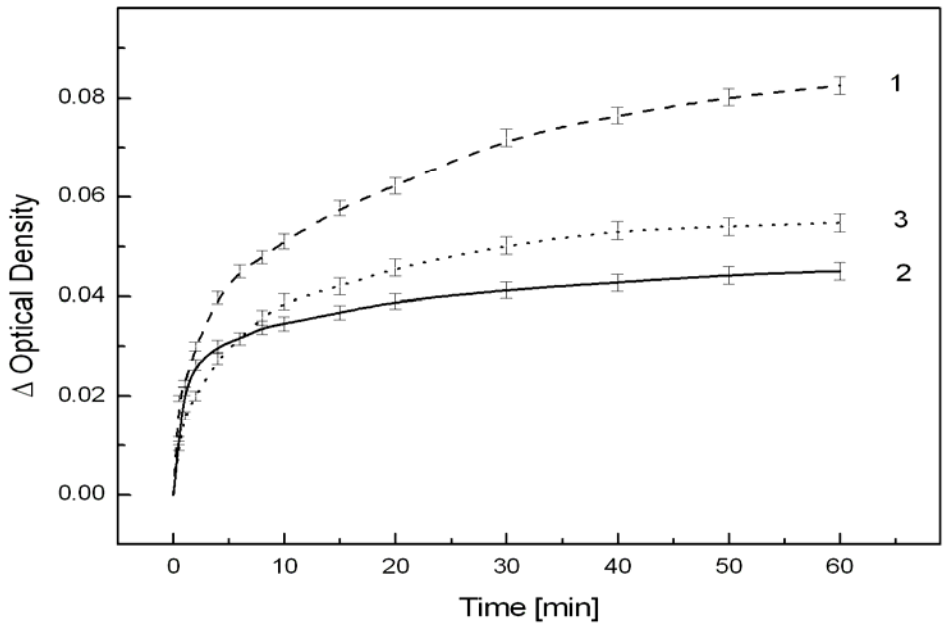

Fig. 5. The time course of bleaching of RNO at 440 nm dependence in the RNO- imidazole-bopindolol- $CuSO_4$- H_2O_2 system (curve 1). Curve 2- the same as curve 1 but in the absence of bopindolol. Curve 3- the same as curve 1 except that 5,5-dimethyl-1,3-cyclohexanodione was added (1mM). The reaction mixture contained 0.08 mM RNO, 60 mM imidazole, 0.5 mM bopindolol, 0.5 mM $CuSO_4$, 0.5 mM H_2O_2, carbonate buffer 0.1 M, pH 9.2. Cell 0.2 cm. Temperature 295 K.

Curve 2 presents the loss of RNO in the $CuSO_4$-H_2O_2 system, which is shown as the decrease of optical density at 440 nm. The loss of RNO is increased in the presence of bopindolol (curve 1). This is in agreement with the observed increased in the ESR signal intensity (Fig. 2). Curve 3 (Fig. 5) presents the quenching effect of the $^1O_2$ quenchers (5,5-dimethyl-1,3-cyclohexano-dione) on the bleaching of RNO in the $CuSO_4$-H_2O_2 system confirming the presence of $^1O_2$ in the reaction.

CONCLUSION

The results of this study demonstrate the enhancing effect of bopindolol on the production of reactive oxygen species during decomposition of hydrogen peroxide in the presence cupper ions. These experiments also confirm the previously proposed mechanism of bopindolol oxidation in the presence of cobalt ions. Since increased generation of oxygen species in the presence of bopindolol was observed, precautions should be considered during bopindolol treatment.

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