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TOXIC EFFECTS AND DETECTION OF OXYGEN FREE RADICALS ON CULTURED ARTICULAR CHONDROCYTES GENERATED BY MENADIONE

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The aim of this work was to study the proliferation pathological perturbations of cultured chondrocytes in response to menadione, an oxygen free radicals producing drug. Rabbit articular chondrocytes in monolayer culture were treated with 10^{-5} M, $1.5 \cdot 10^{-5}$ M and $2 \cdot 10^{-5}$ M of menadione during three days. A dose dependent decrease of the proliferative capacity was observed. Flow cytometry analysis revealed a perturbation of the cell cycle progression consisting in an accumulation of cells in the S and $G_2 + M$ phases. This growth perturbation was due to oxygen radicals production since a treatment with catalase suppressed these toxic effects. Furthermore, to identify oxygen derived radicals in the cellular suspension of cultures treated with menadione, we used a technique of spin-trapping coupled with electron spin resonance (ESR). The ESR signal corresponding to the DMPO hydroxyl radical adduct (DMPO-OH) has been detected. The spectra observation indicated the actual production of hydroxyl radical. However, superoxide anions have not been identified; this fact can be explained by the low reactivity of these anions with DMPO and by the decomposition of signal DMPO-OOH to DMPO-OH.

KEY WORDS: Menadione, cultured chondrocytes, free radicals, catalase, flow cytometry, electron spin resonance.

INTRODUCTION

In response to an oxidative stress, an overproduction of oxygen radical species is observed. These are involved in some pathologic states (inflammation, carcinogenesis, chemical cytotoxicity, articular damages) resulting in damages in cell membranes (lipid peroxidation), proteins, mitochondria and DNA.[1–3]

Many studies have been performed in order to elucidate the molecular mechanism of the oxidative stress. Concerning cartilage degradations, the use of specific cells as experimental models is useful to provide information about the genesis.

Articular chondrocytes in culture treated with an oxygen radical system constitute an interesting model which could approach the articular pathologic conditions *in vivo*.

Menadione, as well as quinones, is known to be cytotoxic by a mechanism involving redox cycling and oxidative stress. Many quinones are thought to be transformed

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through their one-electron reduction to semiquinone radicals which subsequently enter redox cycles with molecular oxygen to produce active oxygen species and oxidative stress.[4,5]

In this study, we report the evaluation of the toxic effects of menadione (2-methyl-1-4-naphtoquinone) and the detection of oxygen free radicals generated on rabbit articular chondrocyte cultures. In order to evaluate the toxic effects of menadione, we investigated the proliferative activity on cell growth parameters and on cell cycle analysis by flow cytometry. On the other hand, to precise the oxygen free radical generation, we studied the antioxidant effects of catalase and α-tocopherol, and the direct observation of this radical production using spin trapping technique coupled with electron spin resonance (ESR) spectrometry.

MATERIALS AND METHODS

Origin and Culture of Chondrocytes

Articular cartilage was removed from the shoulder and knee joints of 1–2 months old *"fauve de bourgogne"* rabbits. Chondrocytes were enzymatically released from cartilage slices using a technique derived from Benya *et al.*[6]

The technique uses successive digestions with trypsin (0.05% w/v, Choay) and collagenase (0.3% and 0.06%, Worthington).[7] For primary cultures, isolated cells were cultured in Ham's F12 medium (Gibco) supplemented with 10% foetal calf serum and gentamicin (4 μg/ml) and maintained at 37°C in an atmosphere of 5% CO_2 in air.

The medium was changed 2 days after cell attachment and confluency was obtained after 6 days of culture. Chondrocytes were then harvested by trypsinization, seeded (7300 cells/cm^2) and this first subculture was used for the study.

Treatment of Cell Culture

Menadione sodium bisulfite (Sigma) was added to the culture medium of subculture 24 hr after seeding. The concentrations 10^{-5} M, $1.5 \cdot 10^{-5}$ M and $2 \cdot 10^{-5}$ M were selected for all experiments to evaluate a dose-effect relation.

Proliferation analysis

Plastic petri dishes (35 mm) were seeded with $7 \cdot 10^4$ cells. After 24, 48 and 72 hr of treatment, cells were collected by trypsinization and cell proliferation was measured using a cell counting method (Hemocytometer). Cell viability was monitored by the cells ability to include neutral red (Fluka) according to the technic of Borenfreund *et al.*[8]

Cell Cycle Analysis by Flow Cytometry

Cells were seeded at $2 \cdot 10^5$ cells (25 cm^2 flask). After 1 day of culture the flasks received fresh medium containing menadione at concentrations chosen (10^{-5} M, $1.5 \cdot 10^{-5}$ M, $2 \cdot 10^{-5}$ M).

Monolayer cultures were harvested after 24 hr by trypsinization and monodispersed

cells were fixed with 70% ethanol. Cell cycle distribution was analyzed by flow cytometry using a technique derived from Crissman *et al.*[9]

DNA content was measured after a simultaneous cell treatment by ribonuclease (Sigma) (0.5 mg/ml in Phosphate Buffer Saline) and staining with propidium iodide (Sigma) (20 μg/ml in PBS). Cells were preserved into ice until the analysis in a Cytofluorograf model FC 200/4800A (Ortho-Instruments). The cell cycle distribution was determined with $2 \cdot 10^4$ cells by using the peak reflect method.[10]

Antioxidant Treatment

Antioxidant effects were measured after 24, 48 and 72 hr of treatment with menadione on cell proliferation.

Chondrocytes, seeded in Petri dishes ($7 \cdot 10^4$ cells/35 mm) and in multidishes ($8 \cdot 10^5$ cells/ml), were incubated with menadione and two antioxidants: Catalase (Sigma), an H_2O_2 scavenger and α-tocopherol (Sigma) which traps hydroxyl radical and peroxyradicals in lipidic peroxidation.

Spin Trapping Study by Electron Spin Resonance (ESR)

The technique of spin trapping makes use of spin trap which reacts with an unstable free radical giving rise to a relatively stable ESR observable free radical adduct.

The spin trap DMPO (5-5-dimethyl-1-pyrroline-N-oxide) (Aldrich), which is lipid soluble, was chosen to react with active oxygen species to yield nitroxide derivatives. Spin adducts have been identified by their spectral characteristics.[11,12]

The ESR spectra were recorded at room temperature using ER 200D Bruker spectrometer operating at 9.76 GHz with a modulation frequency of 100 KHz and an amplitude of 1.0 G. The microwave power was set at 10 mW and the receiver gain was 10^6.

To detect menadione stimulated oxygen species generation, reaction mixtures containing suspensions of cultured chondrocytes were prepared in PBS (Gibco). DETAPAC, (diethylenetriaminepentaacetic acid) (Sigma) was included into the buffer to chelate the transition metal ions (iron) and hence prevent OH· formation by inhibiting the Haber Weiss reaction in the medium.[13]

The reaction mixtures contained 20 μl of DMPO (10^{-1} M), 50 μl of cells (10^6, $3 \cdot 10^6$, $5 \cdot 10^6$/ml), 100 μl of menadione (10^{-2} M, 10^{-3} M) and 30 μl of DETAPAC (10^{-2} M).

After different times of incubation, reaction mixtures were transferred to a flat quartz (200 μl) ESR cell and fitted into the cavity of the spectrometer.

Statistical Analysis

Control and treated cell populations were compared by using the student's t test.

RESULTS

Proliferation Analysis

Proliferation studies (Figure 1) were carried out 24, 48 and 72 hr after treatment with menadione at toxic concentrations (10^{-5} M, $1.5 \cdot 10^{-5}$ M, $2 \cdot 10^{-5}$ M). We have

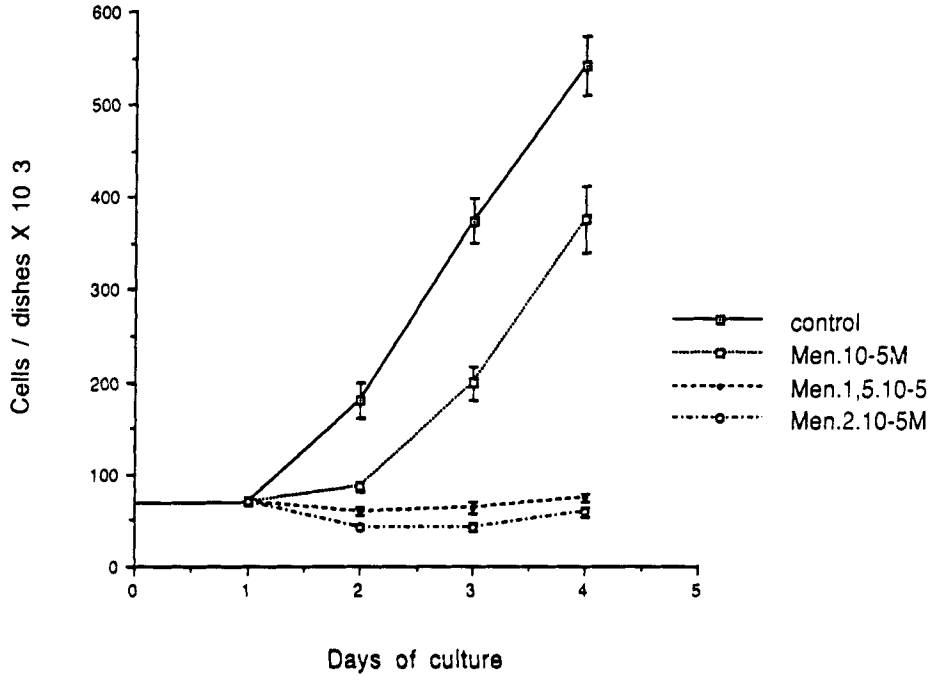

FIGURE 1 Menadione effects on chondrocyte growth. Cells were seeded at $7 \cdot 10^4$ cells/cm^2. 24 h after seeding, cells were treated with menadione (····· 10^{-5} M, ––– $1.5 \cdot 10^{-5}$ M, –·–·– $2 \cdot 10^{-5}$ M). After 24, 48 and 72 hr of incubation, control and treated cells were collected by trypsinization and counted (Hemocytometer).

observed that menadione (10^{-5} M) induced a decrease of cell growth. With the other concentrations ($1.5 \cdot 10^{-5}$ M, $2 \cdot 10^{-5}$ M), cell proliferation was completely inhibited. Viability study (Table I) showed that menadione was cytotoxic on 50% of cells (IC_{50}: Inhibitory Concentration 50) with $2 \cdot 10^{-5}$ M whereas 10^{-5} M and $1.5 \cdot 10^{-5}$ M were not significantly different from the control.

TABLE I
Cell viability studies after menadione treatment. Cells were seeded at $8 \cdot 10^5$ cells/ml in multidishes and treated after 24 hr of culture with menadione (10^{-5} M, $1.5 \cdot 10^{-5}$ M, $2 \cdot 10^{-5}$ M). After 24 hr of treatment, cell viability was monitored with neutral red staining and measured by spectrophotometry

Menadione concentrations	Percentage of cell viability Treated cells/control (± SD)
10^{-5} M	112.2 (± 8.1)
$1.5 \cdot 10^{-5}$ M	111.4 (± 13.2)
$2 \cdot 10^{-5}$ M	53.6 (± 12.3)

TABLE II
Cell cycle analysis of chondrocytes treated with menadione. Cells were seeded at $2 \cdot 10^5$/flask (25 cm^2) and treated after 24 h of culture with menadione (10^{-5} M, $1.5 \cdot 10^{-5}$ M, $2 \cdot 10^{-5}$ M). After 24 h of treatment, cell cycle distribution was analyzed by flow cytometry for control and treated cells

	Cell repartition in the different phases of cell cycle (± SD)		
Treatment	G0/1 phase	S phase	G2 + M phase
Control	59.4 (± 0.6)	22.4 (± 0.4)	18.2 (± 0.9)
Men. 10^{-5} M	55.4 (± 2.9)*	23.3 (± 0.8)	21.3 (± 1.3)*
Men. $1.5 \cdot 10^{-5}$ M	49.7 (± 5.2)**	27.1 (± 1.4)**	23.2 (± 2,4)**
Men. $2 \cdot 10^{-5}$ M	45.1 (± 0.7)***	29.6 (± 0.9)***	25.3 (± 0.3)***

Significance: *$P < 0.05$; **$P < 0.02$; ***$P < 0.01$ by student's t test.

Cell Cycle Analysis

Flow cytometric analysis of DNA content per cell was realized to study the modifications of cell proliferative capacity observed in growth curves. This evaluation was performed after 24 hr of treatment with menadione (10^{-5} M, $1.5 \cdot 10^{-5}$ M, $2 \cdot 10^{-5}$ M). We observed a significant increase in the proportion of cells in the S and G_2 + M phases with a corresponding decrease of cells in the $G_{0/1}$ phase (Table II). A dose dependent effect was observed with an enhancement of 39% of the cells repartition in G_2 + M phase in comparison with the control.

Antioxidant Activities

To evaluate free radical generation, catalase and α-tocopherol effects were assayed on chondrocytes treated with menadione (10^{-5} M, $1.5 \cdot 10^{-5}$ M, $2 \cdot 10^{-5}$ M).

Catalase

Proliferation studies were realized after 24, 48 and 72 hr of concomitant treatment with catalase and menadione. Different concentrations were tested (100, 200, 300 units/ml). The last concentration (300 units/ml) was slightly cytotoxic (91.5% ± 5.7 of cell viability) but no cytotoxic effects were observed after concomitant treatment of cells with menadione (data not shown).[20] We observed on cell proliferation that catalase protected the cells treated with menadione (Table III). With the lowest concentration of menadione after 24 h of treatment, the protective effect of catalase was complete. At higher concentration of menadione, cells were partially protected by catalase.

α-tocopherol

The study was carried out with α-tocopherol (10^{-5} M) in the same conditions as catalase, we observed a non significative protection (data not shown).

Spin Trapping Measurement by ESR

Spin-trapping using DMPO was employed to directly observe the free radical generation produced by menadione in contact with a suspension of cultured

TABLE III

Antioxidant effects of catalase on chondrocyte growth after menadione treatment. Cells were seeded at $7 \cdot 10^4$ cells/cm^2. After 24 h of seeding, chondrocytes were treated with menadione (10^{-5} M, $1.5 \cdot 10^{-5}$ M, $2 \cdot 10^{-5}$ M) and catalase (100 U, 200 U, 300 U). After 24, 48 and 72 hr of incubation, control and treated cells were collected by trypsinization and counted (Hemocytometer)

Menadione (M) and catalase (U) concentrations	Percentage cells treated/control (+ SD)		
	24 hr	48 hr	72 hr
Catalase 100	98.4 (± 14.3)	77.8 (± 6)	101.8 (± 0.9)
Catalase 200	96.4 (± 8.8)	75.9 (± 3)	58.1 (± 0.8)
Catalase 300	74.2 (± 6.8)	38.4 (± 2.2)	28.9 (± 3.2)
Menadione 10^{-5}	45.8 (± 0.9)	42.4 (± 0.8)	76.8 (± 1.1)
Men. 10^{-5}, Cat. 100	98.7 (± 3.2)	96.1 (± 4.6)	111.7 (± 5.4)
Men. 10^{-5}, Cat. 200	122 (± 4.3)	78 (± 5.4)	106.9 (± 5.4)
Men. 10^{-5}, Cat. 300	109.8 (± 12.7)	62.8 (± 7.3)	56.5 (± 1.2)
Menadione $1.5 \cdot 10^{-5}$	13.8 (± 0.5)	9.3 (± 2.2)	12.6 (± 1.1)
Men. $1.5 \cdot 10^{-5}$. Cat. 100	64.9 (± 4.5)	57.5 (± 3.7)	63.3 (± 2.7)
Men. $1.5 \cdot 10^{-5}$. Cat. 200	88.9 (± 10.5)	60.4 (± 4.3)	70.5 (± 2.9)
Men. $1.5 \cdot 10^{-5}$. Cat. 300	65.3 (± 7.3)	41.8 (± 5.6)	46.8 (± 2)
Menadione $2 \cdot 10^{-5}$	8.5 (± 0.7)	5.1 (± 0.6)	7.1 (± 0.2)
Men. $2 \cdot 10^{-5}$. Cat. 100	22.6 (± 3.1)	10.8 (± 0.2)	15.2 (± 0.3)
Men. $2 \cdot 10^{-5}$. Cat. 200	29.8 (± 4.2)	24.5 (± 2)	29.3 (± 1.8)
Men. $2 \cdot 10^{-5}$. Cat. 300	41.9 (± 1.1)	30.4 (± 3.4)	31.2 (± 2.2)

chondrocytes. Experiments were carried out on different cells concentrations (10^6, $3 \cdot 10^6$ and $5 \cdot 10^6$/ml) treated with menadione (10^{-3} M, 10^{-2} M). High concentrations of cells and menadione were selected to obtain observable ESR spectra. Kinetic studies were performed.

When chondrocytes were exposed to menadione (10^{-3} M), we observed an ESR spectra with a signal corresponding to the DMPO hydroxyl radical adduct (DMPO-OH) characterised by its 1:2:2:1 peaks.[13–15] After 5 mn of incubation (Figure 2), signals were detected but the spin adducts could not be identified because of their low amplitude. After 15, 30, 60 mn of treatment, the DMPO-OH signal clearly appeared with an amplitude increasing with various time. With cells concentrations, the observed ESR signal intensity appeared to be proportional to the cells number (data not shown).

When cells (10^6/ml) were treated with a higher concentration of menadione (10^{-2} M), we clearly observed the apparition of the DMPO-OH signal after 5 mn of incubation (Figure 3). The signal amplitude increased with time, being 5 fold greater after 60 mn of treatment. No ESR signal was observed with the controls.

DISCUSSION

These studies have displayed that menadione at concentrations of 10^{-5} M, $1.5 \cdot 10^{-5}$ M and $2 \cdot 10^{-5}$ M produces a decrease in chondrocyte proliferation with a dose dependent effect.

Cell viability experiments have shown that menadione was not cytotoxic at 10^{-5} M and $1.5 \cdot 10^{-5}$ M whereas at $2 \cdot 10^{-5}$ M, it was cytotoxic on 50% of cells (IC_{50}). At

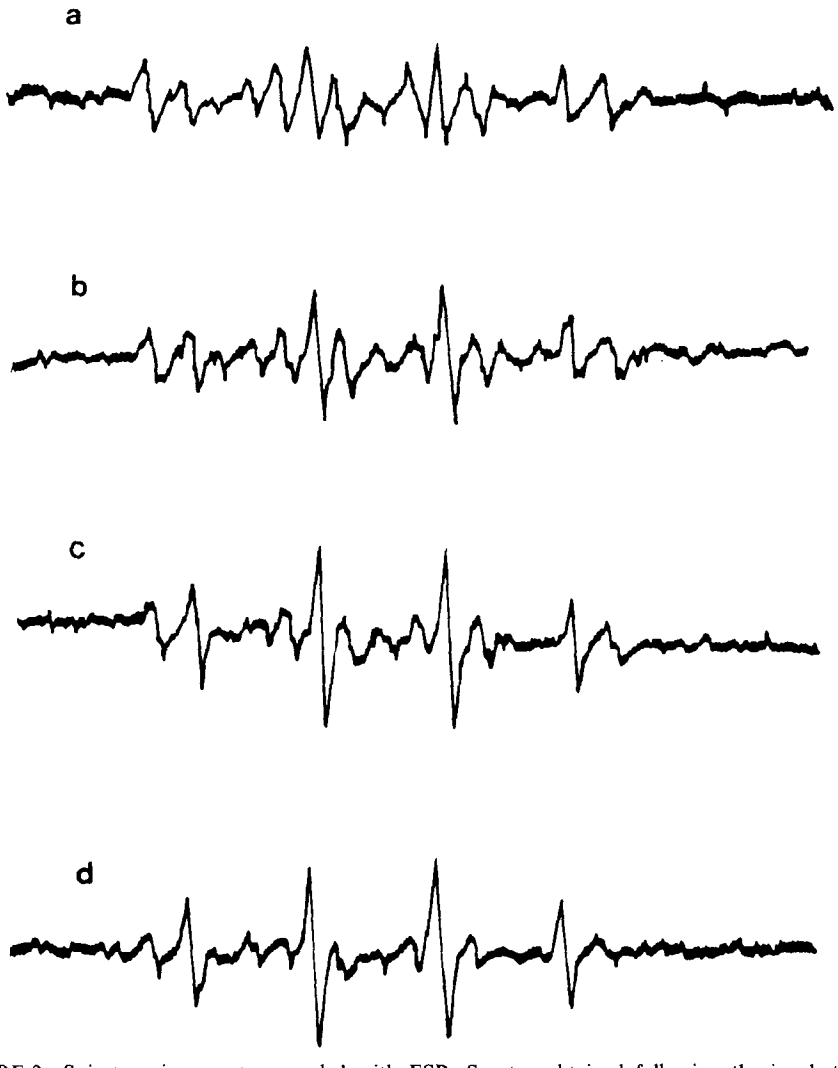

FIGURE 2 Spin-trapping spectra coupled with ESR. Spectra obtained following the incubation of chondrocytes (10^6/ml) with menadione (10^{-3} M) in the presence of DMPO (10^{-1} M), DETAPAC (10^{-2} M) in phosphate buffered saline. Time of incubation was (a): 5 mn, (b) 15 mn, (c) 30 mn, (d) 60 mn. Microwave power was 10 mW, the modulation frequency was 100 KHz with an amplitude of 1.0 G. The receiver gain was 10^6.

non cytotoxic concentrations of menadione, endogenous protective mechanisms of chondrocytes probably neutralize the small amount of generated free radicals. However, at higher concentrations, the free radicals generation exceeds the capacity of the protective mechanism and leads to the cells death.[1]

Analysis of the DNA content per cell by flow cytometry has permitted to us the study of the perturbation of cell proliferative capacity observed on growth curves. This

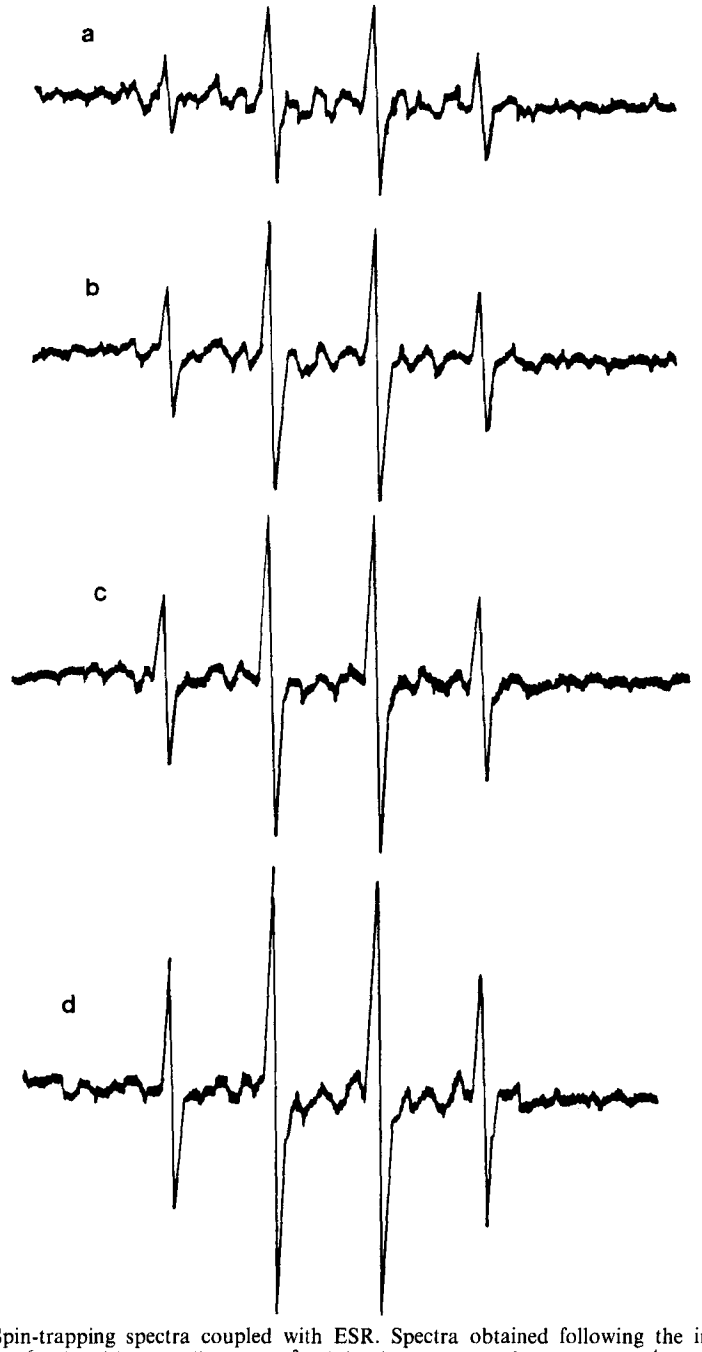

FIGURE 3 Spin-trapping spectra coupled with ESR. Spectra obtained following the incubation of chondrocytes (10^6/ml) with menadione (10^{-2} M) in the presence of DMPO (10^{-1} M), DETAPAC (10^{-2} M) in phosphate buffered saline. Time of incubation was (a) 5 mn, (b) 15 mn, (c) 30 mn, (d) 60 mn. Microwave power was 10 mW, the modulation frequency was 100 KHz with an amplitude of 1.0 G. The receiver gain was 10^6.

study indicated that menadione modified cell repartition in the cell cycle leading to an accumulation of cells in the S and $G_2 + M$ phases. Similar results have been observed after treatment with compounds that induce free radical or lipid peroxidation reactions on cell culture. Studies with cumene hyperoxide, 4-hydroxynonenal, desferrioxamine and hypoxanthine plus xanthine oxidase carried out on the cell cycle led to an accumulation or an arrest of cells in the $G_2 + M$ phase.[3,16,17]

Investigations on inducing G_2 arrest by oxygen free radicals drugs have shown that these events could be associated with DNA base damage, strand breakage and modification in genes associated with cell growth control expression.[16,18]

Antioxidants effects on cell proliferation have shown that catalase has an important protective effect on cells treated with menadione indicating that free radicals are implicated in this phenomenon. Previous works have observed that the abolition of the cytotoxic effect produced by different systems generating free radicals suggested the involvement of hydrogen peroxide in the extracellular compartment.[19,20]

We observed with the lowest concentration of menadione, a complete protection of catalase. Free radicals generated in the cell could be neutralized by the endogenous protective mechanisms of chondrocytes and extracellular production of hydrogen peroxide is inhibited by catalase. At higher concentrations of menadione, cells were partially protected. We suppose that higher intracellular production of free radicals could exceed the endogenous protective mechanism and/or that higher extracellular production of hydrogen peroxide could not be completely neutralized by catalase.

Dicker *et al.*,[21] who worked on the menadione effects on hepatic microsomes after ethanol consumption, found an increase in $\cdot OH$ generation which was inhibited by catalase, implicating H_2O_2 as the precursor of $\cdot OH$.

Moreover, Pigeolet *et al.*,[1] have observed that catalase was inactivated by superoxide anions and by hydroxyl radical whereas organic peroxides had no effect. Studying the mechanism of this inhibition, they suggested the possibility of hydroxyl radicals formation from the Fenton reaction with iron directly present in the catalytic site of catalase. The same mechanism would also explain the inhibition obtained with the superoxide anions. However, this inactivation was slight, probably because of the low accessibility to the active site.

Free radical generation detected by the catalase antioxidant effect was directly observed in cells suspensions treated with menadione using electron spin resonance spectroscopy. Spin-trapping allowed the detection of the DMPO-OH adduct signal characteristic of the hydroxyl radical. Superoxide anions have not been identified. Kinetic studies showed that the ESR signal observed, reached a maximum after 60 mn of treatment and appeared to be proportional to the menadione and cell concentration.

Rosen *et al.*[13] and Gant *et al.*[5] working on endothelial cells and hepatocytes treated with menadione, detected signals of DMPO-OOH and DMPO-OH. Observation of superoxide anions (DMPO-OOH) was explained by the biological reduction of menadione in cell to a semiquinone, which then reacts with oxygen yielding superoxide. To explain this reaction, studies have been carried out in order to distinguish intracellular from extracellular sites of radicals formation.[13,22]

Rosen *et al.*[13] found that superoxide does not diffuse across the plasma membrane but is rather produced at both intracellulary and extracellulary mediums. They firstly proposed that intracellular reductases can reduce menadione to semiquinone, which then transfer an electron to oxygen, yielding superoxide. Secondly, menadione can be reduced by cellular DT diaphorase to 2-methyl-1-4-dihydronaphthalene, which can diffuse across cell membranes in the extracellular compartment and reduce

menadione, yielding menadione semiquinone by disproportionation. Reduction of oxygen by the semiquinone generates superoxide anions. Similar results were found by Hassett *et al.*[23] working on paraquat.

Several reports have shown that the observation of the hydroxyl radical signal (DMPO-OH) following the production of superoxide anions can be explained by transformation of the $O_2 \cdot^-$ in H_2O_2 then $OH \cdot$ with iron (Fe^{3+}) or by decomposition of DMPO-OOH in DMPO-OH.[13–15,22,24]

Yamazaki *et al.*,[11] working on paraquat measured the conversion ratio between DMPO-OOH and DMPO-OH. They found a ratio of 2.8% indicating an actual production of hydroxyl radical. The predominant observation of the hydroxyl radical signal can be explained by the fact that the DMPO-OH signal is very stable while the DMPO-OOH signal disappears after 10 mn of reaction.[11,13,14]

Kinetic analysis carried out by Yamazaki *et al.*[11] showed that the DMPO-OH signal amplitude is nearly equal to the total amount of DMPO-OH accumulated during the entire reaction while the total amount of formed DMPO-OOH is more important than that measured by spin trapping. The constant reaction rate of DMPO indicates that the hydroxyl radical reacts rapidly with DMPO while the superoxide anions have a slow reaction rate.

These results allow us to think that menadione interacts with cultured chondrocytes producing an alteration of the cell proliferative capacity associated with a cell accumulation in the S and $G_2 + M$ phase. Antioxidant treatment has shown that this toxic effect is a result of free radical generation. Spin trapping has permitted to observe an actual production of hydroxyl radical. Superoxide anions have not been identified because of the low reactivity with DMPO and the decomposition of the signal DMPO-OOH to DMPO-OH.

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