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DEVELOPMENT OF A SIMPLE ANTIOXIDANT SCREENING ASSAY USING HUMAN SKIN FIBROBLASTS

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The purpose of this study was to develop a simple antioxidant screening assay for quantifying the protective effects of antioxidant enzymes, inhibitors and scavengers against extracellularly generated oxygen species on human skin fibroblast cytotoxicity. Different *in vitro* oxidative stresses have been studied: xanthine oxidase–hypoxanthine, flavin mononucleotide–NADH, and hydrogen peroxide. Cytotoxicity and protection were evaluated by two procedures: evaluation of the living cells using a colorimetric method (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide MTT), and ability of the viable cells to adherate and proliferate. Hypoxanthine–xanthine oxidase and H_2O_2 induced a dose dependent cytotoxicity only when we considered the delayed toxicity. The influence of the cell density was also investigated. The delayed toxicity was higher when cell density increased. One hundred percent protection against free radical cytotoxicity induced by the three systems were obtained with catalase (500 U/ml). When the oxidative stress used was H_2O_2 90–96% protection was obtained with deferoxamine an iron chelating agent that prevents iron catalysed radical reactions. Using the colorimetric method no significant protection was obtained when SOD was added before and during the stresses. Using the fibroblasts ability to proliferate SOD (10–150 μg/ml) reduced xanthine oxidase (20 U/l)-hypoxanthine (0.10–0.30 mM) or H_2O_2 (1–6 mM) cytotoxicity by 15–20%. SOD did not act as antioxidant when the applied stress was mediated by flavin. In this study we showed a paradoxical effect and the cytotoxicity of flavin–NADH system increased when we added SOD to the cell medium. This simple and reliable antioxidant screening assay required no costly or radioactive equipment.

KEY WORDS: Human skin fibroblasts, oxidative stress, xanthine oxidase, hydrogen peroxide, flavin, antioxidant assay.

INTRODUCTION

Free radicals can be generated in living organisms by different pathological pathways, including ultraviolet radiations,[1] phagocytosis, ischemia,[2] cell activation due to the defect of the biocompatibility of various compounds such as hemodialysis membranes,[3] etc. Therefore a number of different cell types, e.g., polymorphonuclear cells, monocytes or macrophages, and endothelial cells, can release oxygen free radicals into the bloodstream or in various tissular targets reached after diapedesis.[4,5] The superoxide anion (O_2^-), one of the main oxygen free radicals generated by cells, participates in chain reactions producing hydroxyl ($OH^\cdot$), peroxyl ($ROO^\cdot$), alkoxyl

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($RO^{\cdot}$) and hydroperoxyl radicals ($HOO^{\cdot}$), which can damage cells.[6] These reduced oxygen species, which in turn have been shown to inhibit enzyme systems, react with membrane constituents and nuclear contents, deplete cells of key metabolic intermediates, and can ultimately cause cell death.[7] Protection against these reactive oxygen species is provided by enzymatic systems such as catalase, superoxide dismutase (SOD) and glutathione peroxidase (GSHPx)[8,9] and by molecules like tocopherol, carotene, ascorbic acid and glutathione.[10–12]

Many studies *in vivo* and *in vitro* have demonstrated the protective effects of these defence systems[13] but it is difficult to know and to compare their exact roles in different kinds of oxidative stress. The aim of the present work was to develop experimental procedures for the use of a simple cell model, the fibroblast, to evaluate the toxicity of oxygen free radicals and the efficiency of antioxidant molecules. The protective effects of superoxide dismutase (SOD), catalase and deferoxamine towards cell viability were evaluated by the use of three different oxygen free radical-producing systems: hypoxanthine–xanthine oxidase (HX–XO), flavin mononucleotide–nicotinamide adenine dinucleotide reduced form (FMN–NADH) and hydrogen peroxide (H_2O_2).

MATERIAL AND METHODS

Chemicals

RPMI 1640, fetal calf serum (FCS) and L-glutamine were purchased from Gibco (Grand Island, USA); penicillin, streptomycin and kanamycin were from Boehringer (Mannheim, Germany) and fungizone from Squibb (Princetown, USA). Superoxide dismutase (Zn–Cu SOD), catalase, xanthine-oxidase (XO), hypoxanthine (HX), flavin mononucleotide (FMN), nicotinamide adenine dinucleotide reduced form (NADH), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma Chemical Co. (Saint Louis, MO, USA). Deferoxamine was purchased from Ciba-Geigy (Rueil Malmaison, France). Hydrogen Peroxide and other chemicals came from Prolabo (Paris, France).

Cell Culture

Human diploid fibroblasts were obtained by biopsies of healthy skin from shaved forearms. The cells were grown in RPMI 1640 medium containing 10% fetal calf serum and supplemented with fungizone (0.5 mg/l), kanamycin (54 mg/l), penicillin (172 000 U/l) and streptomycin (172 mg/l). Fibroblasts were grown to confluence in 75 cm^2 culture dished (Falcon® Plastics, CA, USA) at 37°C under a water-saturated sterile atmosphere containing 6% CO_2 (Forma Scientific Incubator, Marietta, USA). All experiments were done on subcultures between the fifth and tenth transfer.

For the assay, the cells were placed after trypsination in 35 mm diameter culture dishes or in 96 well microplates and incubated for 5–6 days (dishes) or 24–48 h (microplates) at 37°C in 6% CO_2 before studying cytotoxicity. The cell density used were 5×10^5 cells per dish and $2.5–3 \times 10^4$ cells per well at the moment of experimentation.

Evaluating Cell Viability

Cell survival was determined with a rapid modified MTT colorimetric method

described by Mosmann.[13] MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is a yellow tetrazolium salt which is transformed to blue–black formazan by the action of dehydrogenases in active mitochondria. The test is thus an indication of living cells. MTT was dissolved in physiological Dulbecco phosphate buffer (PBS) (5 mg/ml) and filtered through 0.2 μm membranes to remove all insoluble residue and suspended particles. Just before use, the MTT solution was diluted 6 times in Tyrode buffer.[14] After the oxidant test, the plates were rinsed twice with Tyrode buffer (0.100 ml/well) and each well received 0.100 ml of diluted MTT. The cells were incubated for 2 h at 37°C in 6% CO_2, the wells were emptied by inverting and the formazan crystals were dissolved in 0.100 ml of DMSO. Absorbance was determined at 570 nm with a Multiscan spectrophotometer (Titerlek Multiskan Plus, Labsystems Group, Les Ulis, France). It is directly proportional to the number of living cells (calibration curve: 1500 to 75 000 cells per well).

Evaluation of Proliferation Capacity

After the oxidative stress, cells grown in 35 mm diameter Petri dishes were rinsed with Tyrode buffer, treated with trypsin and reincubated for 18 h in fresh culture medium. Non-adhering cells and cell debris were removed by rinsing three times with 0.9% NaCl. Survival levels and proliferation capacities were determined by assaying total protein with the method of Shopsis and Mackay.[15] A calibration curve was prepared with 2.5×10^4 to 10^6 cells per dish. The protein concentrations measured were directly proportional to the number of adherent cells.

Generation of Reactive Oxygen Species

Hypoxanthine–xanthine oxidase assay Xanthine oxidase (XO) and hypoxanthine (HX) were prepared in Tyrode buffer, pH 7.4. The HX–XO mixture was incubated with the cells for 90 min, after which the cells were either immediately treated with trypsin and reincubated for 18 h in fresh culture medium or held for an additional 90 min incubation in Tyrode buffer (without reaction mixture) before adding trypsin. The first case determined the immediate toxicity of the HX–XO system, while the second determined delayed toxicity.

In every experiment, untreated control cells were incubated in the presence of the same buffer and treated with trypsin in the same conditions as described above. The toxicity of the reaction mixture was investigated by varying the HX concentrations (0.065–0.20 mM) with a constant XO concentration of 25 U/l, as well as by varying the XO concentration (20–25 U/l).

Hydrogen peroxide The cytotoxic effect of hydrogen peroxide was measured by incubating the reaction mixture with cells in darkness for 30 min. The H_2O_2 solution was prepared just before use in PBS buffer without phenol red. The concentration of the stock solution was verified spectrophotometrically ($\lambda = 230$ nm; $E = 81\ M\ cm^{-1}$) (Uvikon Spectrophotometer 860; Kontron Instruments, Rotkreuz, Switzerland). After the stress, cells were washed twice with PBS, buffer, treated with trypsin, and reincubated in fresh medium.

Flavin mononucleotide-NADH system The method of Michelson[16] was used to produce O_2^- by photoreduction of flavin (FMN). Different doses of FMN/NADH were

used (0.05–0.2 mM) in equimolar ratios. The reaction mixture was prepared just before use in Tyrode buffer. Two ml of reaction mixture were added to each 35 mm diameter dish. The preparation was then irradiated with 0.02 J/cm^2 of UVA (λ_{max} = 375 nm) (UVASUN 2000, Mutzhas Co, Munich, Germany). According to Michelson,[16] the quantities of singlet oxygen (O_2^*) produced in these conditions are minimized. Reoxidation of dihydroflavine by an electron donor such as NADH facilitates the production of superoxide radicals (O_2^-) by the photoreduction of FMN.

Antioxidant enzymes and scavengers SOD (3150 U/mg protein) and catalase were prepared in Tyrode buffer and incubated with cells 15 min before the induction of stress. Dimethyl sulfoxide 0.1 M, mannitol 0.1 M and deferoxamine 1 mM were used in the same conditions. Each antioxidant remained in contact with the cells during the stress.

Processing the Results

The toxic effect of a stress was evaluated by assaying the indicator parameter chosen (total protein or absorbance of formazan) in each dish, using the following equation:

$$X = [(\text{control plates} - \text{exposed plates})/\text{control plates}] \times 100$$

The protective effect of an antioxidant was determined with the following equation:

$$\% \text{ protection} = (X - Y/X) \times 100,$$

where X is defined as above and Y is:

$$Y = [(\text{treated unexposed plates} - \text{treated exposed plates})/ \times \text{treated unexposed plates}] \times 100$$

Statistics

The results are presented as mean $\pm$ 1 SD (standard deviation) and data were analysed by a Mann-Whitney U-test.

RESULTS

Cytotoxic Effect of Free Radicals Generated by the HX–XO System

Oxidative stress caused by HX–XO resulted in cell death which increased with increasing enzyme concentrations and with increasing substrate concentration. Toxicity measured immediately after stress induction was not the same as that measured 90 min later: immediate toxicity was much greater than delayed toxicity, with an abrupt break in the slope at doses between 2.5 and 10 U/l (Figure 1).

The action of antioxidant enzymes added to the cells 15 min before the stress decreased the toxic effects of the system (Figure 2). Protection by catalase reached 100%, while that by SOD was very low, regardless of the intensity of the stress. Higher concentrations of SOD did not improve cell protection. An apparent plateau was reached at SOD doses of about 75 μg/ml.

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) method enabled smaller quantities of cells to be used. As can be seen (Figure 3), toxicity was

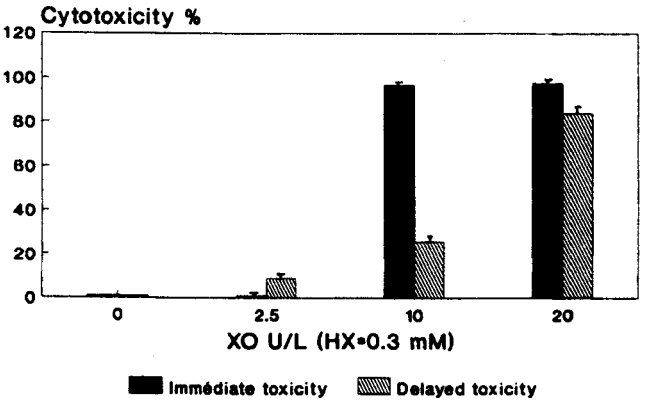

FIGURE 1 Influence of the oxidative stress conditions on the cytotoxicity of the hypoxanthine–xanthine oxidase system towards human fibroblasts. The cells were trypsinized immediately after radical exposure (immediate toxicity) or 90 min later (delayed toxicity). Values represent mean ± 1 SE of three experiments.

a function of the number of cells per well. In order to increase the sensitivity of the method a density of about 2.5×10^4 cells per well was found to be optimal. The comparison of both methods for evaluating cell responses showed that the slopes were similar when the delayed toxicity method was used (Figure 4).

When studying antioxidant enzymes, the results obtained by determining cell viability with the MTT method were not the same as those obtained by measuring total cell protein on the survival adherent cells 18 h after radical exposure. The antioxidant effect of catalase was very high, while that of SOD was not detectable with the MTT method (Figure 5).

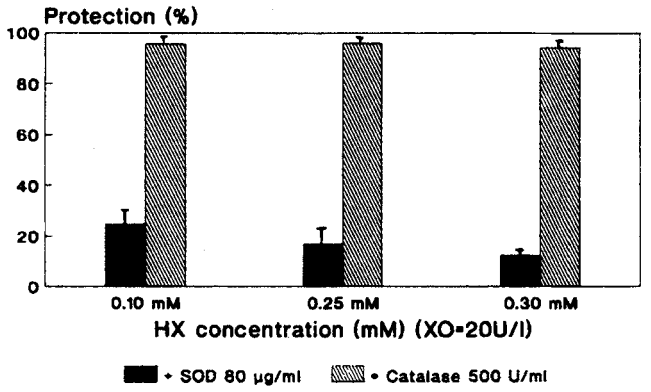

FIGURE 2 Superoxide dismutase and catalase protective effect on the cytotoxicity (as indicated by total protein determined on the survival adherent cells 18 h after radical exposure) of the hypoxanthine–xanthine oxydase (20 U/l) system towards human fibroblasts. The enzymes were added to the tyrode buffer 15 min before the experiment. Each bar indicate mean ± 1 SE (n = 3).

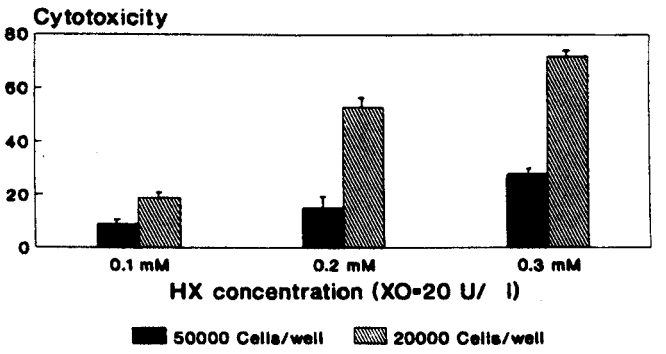

FIGURE 3 Influence of the cell density on the cytotoxicity (as indicated by optical density in the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) test 90 min after exposure) of the hypoxanthine–xanthine oxydase (20 U/l) system towards human fibroblasts (n = 3).

Cytotoxic Effect of Free Radicals Generated by the FMN/NADH System

The FMN–NADH system was highly toxic for cultured fibroblasts. As in the preceding case, this toxicity was greater when determined immediately after the stress (Figure 6). Cell viability measured with the MTT method was not the same as that determined by measuring total protein. Viability was not correlated with the stress applied. In subsequent work, the proliferation capacity of cells was quantified by assaying total proteins.

The antioxidant metalloenzymes SOD exhibited a paradoxical effect: cell mortality increased with increasing quantities of SOD added to the medium (Figure 7), regardless of the FMN concentration used to obtain the oxidative stress. Catalase (500 U/ml) protected cells 100% whereas deferoxamine did not act as a protector (Figure 8).

H_2O_2 Cytotoxic Effect

The toxicity of hydrogen peroxide on cultured fibroblasts was determined with a

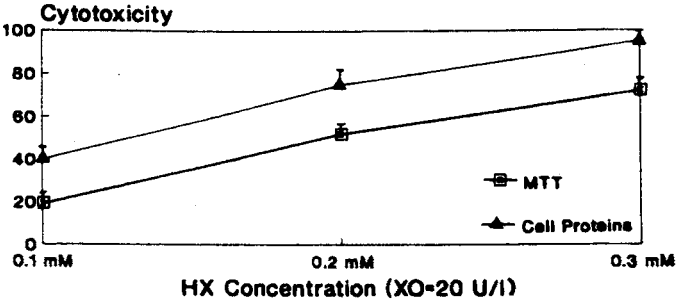

FIGURE 4 Hypoxanthine–xanthine oxidase mediated fibroblasts injury. The cytotoxicity was evaluated by two procedures: cell survival was quantified by optical density in the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenly tetrazolium bromide (MTT) test 90 min after exposure, cell viability and proliferation was measured as total protein (trypsination was done 90 min after exposure and the survival adherent cells were re-fed with fresh medium during 18 h). Each value was the mean of three replicates ± 1 SD.

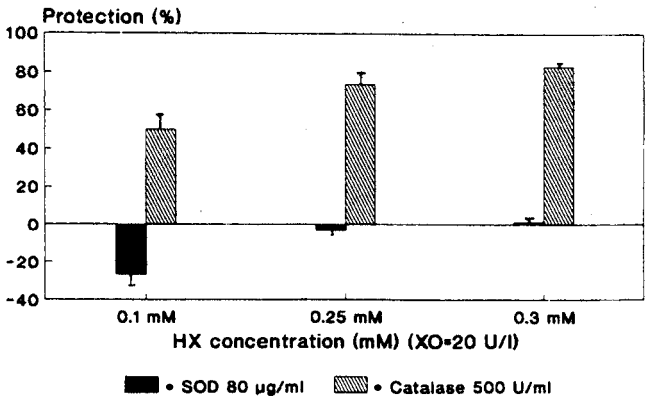

FIGURE 5 Superoxide dismutase and catalase protective effect on the cytotoxicity (as indicated by optical density in the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) test 90 min after radical exposure) of the hypoxanthine–xanthine oxydase (20 U/l) system towards human fibroblasts. The enzymes were added to the tyrode buffer 15 min before the experiment. Each bar indicate mean ± 1 SE (n = 3).

similar method. Increasing doses of H_2O_2 between 10^{-5} M and 10^{-1} M led to increasing toxicity, reaching 100% for 10^{-1} M H_2O_2. The lethal dose 50 (LD50) in this system was about 5×10^{-3} M H_2O_2.

Among the extracellular antioxidant enzymes studied, catalase (500 U/l) led to 100% protection (Figure 9). This work was extended by studying the scavenger action of mannitol and DMSO. The results showed that 0.1 M DMSO protected against H_2O_2-mediated stress, while mannitol at the same concentration exerted a protective effect to a lesser degree. The use of deferoxamine 1 mM as an iron chelating agent led to a protection from 90 to 96%.

DISCUSSION

This study confirms that oxidative stress generated *in vitro* can damage cultured

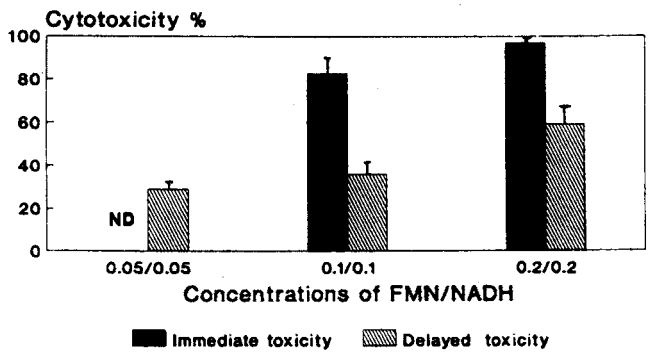

FIGURE 6 Influence of the oxidative stress conditions on the cytotoxicity of the flavin–NADH system towards human fibroblasts. The cells were trysinized immediately after radical exposure (immediate toxicity) or 90 min later (delayed toxicity). Values represent mean ± 1 SE of three experiments.

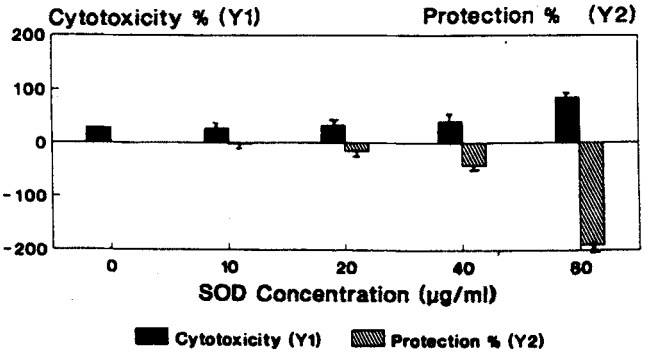

FIGURE 7 Influence of superoxide dismutase concentration on the cytotoxicity (as indicated by total protein determined on the survival adherent cells 18 h after radical exposure) of the flavin–NADH system (0.05 mM) towards human fibroblasts. Values represent mean ± 1 SE of three experiments. Protection index was calculated with the equation $[(X - Y)/X] \times 100$ (see text).

human fibroblasts. In order to determine the role of these reactive oxygen species in the mortality of cells and in their proliferation capacity, antioxidant metalloenzymes (or scavengers), specifically active against O_2^-, H_2O_2 or $OH^\cdot$ were added to the medium 15 min before the stress, according to the method of Tyrell[17] different methods have been used to detect the viability or membrane integrity of cells, including the release of chromium 51 (^{51}Cr) by damaged cells,[18] release of intracellular enzymes, e.g., lactate dehydrogenase,[19] colorimetric methods using trypan blue or MTT,[20] enumeration of colony-forming cells.[17] In the present work, the results obtained with a colorimetric method evaluating cell viability were compared to those obtained by measuring the adhesion and proliferation capacity of cells after radical exposure. An

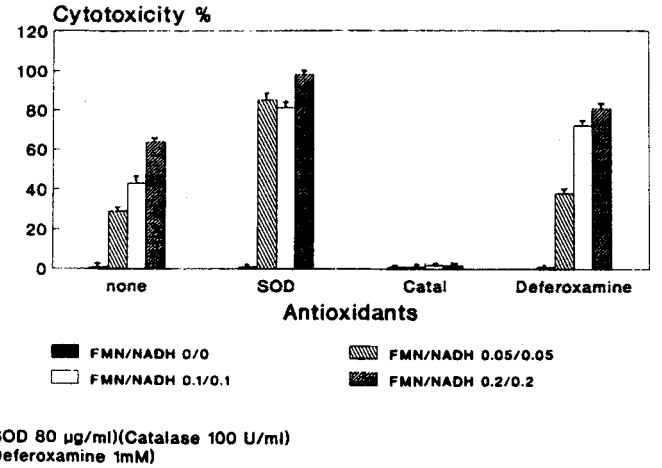

FIGURE 8 Superoxide dismutase, catalase and deferoxamine protective effect on the cytotoxicity (as indicated by total protein determined on the survival adherent cells 18 h after radical exposure) of the flavin–NADH system towards human fibroblasts. The antioxidants were added to the tyrode buffer 15 min before the experiment. Each bar indicate mean ± 1 SE ($n = 3$).

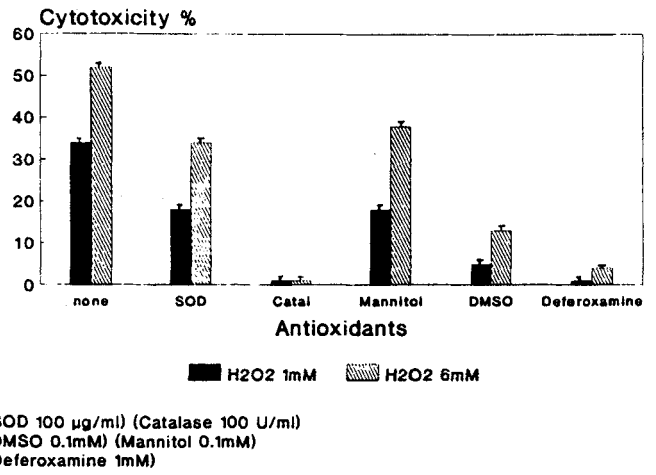

FIGURE 9 The effects of enzymes, scavengers and iron chelating agent on the cytotoxicity of H_2O_2 towards human fibroblasts *in vivo* (as indicated by total protein determined on the survival adherent cells 18 h after H_2O_2 exposure). Superoxide dismutase (100 µg/ml), catalase (500 U/ml). Deferoxamine 1 mM, mannitol 0.1 M or DMSO 0.1 M was added to the system 15 min before the experiment. Values represent mean ± 1 SE of three replicates.

inorganic buffer was used in this work in order to minimize the scavenger and catalase effects of serum.[20,21] When experimentation needed long incubation period (XO–HX, FMN–NADH) we used tyrode buffer which is a carbonate buffer. When cells were not incubated (H_2O_2) we used PBS buffer.

Using the xanthine oxidase-hypoxanthine system, the intensity of cytotoxic effects evaluated with the MTT method were parallel to those determined by measuring cell proliferation capacity with the delayed toxicity protocol. It was found that the application of an oxidative stress to cultured cells rendered them highly sensitive to the effects of trypsin. When trypsin was applied immediately after the stress, damaged cells could no longer adhere to the culture support, leading to a cytotoxic effect close to the law of all-or-nothing. This difference between immediate and delayed toxicity had been described by other authors. Thus, Noel-Hudson[20] showed the delayed toxic effect of HX–XO by measuring cell density 48 h after exposing the cells to a stress. This delayed effect appeared only with high doses of HX and it was suggested that long term damage by oxygen free radicals was involved. At low doses, the inverse phenomenon occurred, i.e., increased cell proliferation, results later confirmed by Murell.[22] In the experimental protocol used here, delayed toxicity depended on the intensity of the applied stress.

The use of the antioxidant metalloenzymes SOD and catalase has confirmed the work of Kellog and Fridovich,[23] who showed that the HX–XO system generates reactive oxygen species i.e., O_2^- (radical) and H_2O_2 (nonradical). In our model of evaluating cell proliferation capacity, the protective effect of SOD varied from 10 to 25%, depending on the intensity of the stress, if the induced stress was intense, the protective effect decreased. In addition, the partial protective effect of SOD was not proportional to SOD doses added to the medium. It may thus be concluded that a small proportion of the toxic effects of the xanthine system are due to radical attack

by the superoxide anion. When we revealed by the MTT method, SOD had no protective effect, in agreement with other published results.[20,21,24] Probably MTT as other tetrazolium salt reacted with O_2^- making this method inappropriate. Catalase protected cells from all lethal effects of the HX–XO system regardless of the method used to measure the toxic effect and confirm the role of H_2O_2 in the toxicity of the HX–XO system. These results agree with the diffusible H_2O_2 molecule causing damage far from its site of production. Such damage involves cell membranes, deoxyribonucleic acid (DNA) and cytoplasmic components. These results were confirmed by our work on H_2O_2 added to the extracellular medium. Toxicity measured after applying a 30 min stress showed that the cytotoxic effect of H_2O_2 is proportional to the intensity of the applied stress. The use of buffer without fetal calf serum enabled the H_2O_2 concentration to be maintained constant throughout the stress. In the present method, catalase protected 100%, which was not the case for mannitol and DMSO, tending to confirm that the hydroxyl radical is less toxic when it is generated in the extracellular medium. Its lifetime is too short to cause cell damage. Its *in situ* production from hydrogen peroxide inside the cell could explain the toxicity of stress mediated by the HX–XO system and that mediated by hydrogen peroxide itself. The addition of deferoxamine protected the cells from H_2O_2 toxicity. This result suggests that this new simple procedure can be used to study Fenton chemistry.

In the present work, we attempted to determine the cytotoxic effect of a system generating O_2^- as described by Michelson,[16] i.e. the photoreduction of flavin in the presence of NADH at 365 nm. As before, the immediate toxicity of free radicals generated by this system was much greater than delayed toxicity. In contrast to results obtained with the HX–XO system, there was no parallelism between cell viability measured with the MTT method and the proliferation capacity of cells measured 18 h after the stress. The determination of antioxidant activities of SOD and catalase showed a paradoxical effect of SOD: the toxicity of the generating sytem increased with increasing doses of SOD. According to Jadot,[25] a complex series of radical reactions could be involved, utilizing the superoxide anion to generate a chain of other radicals. According to Gutteridge[4] the increased H_2O_2 generation in the presence of SOD might produce extra damage by a different mechanism perhaps involving Fenton Chemistry but deferoxamine did not protect the cells in the FMN/NADH system whereas it offered a 90–96% protection when H_2O_2 was applied on the cells. These hypotheses would explain the paradoxical effect of SOD and the protective effect of catalase. It could also explain why the slopes of cell viability and cell proliferation in this system are not similar.

In conclusion, the fibroblast is a useful cell model for studying radical stress. The results of cell adhesion and proliferation capacities after an oxidizing stress may differ from those concerning cell viability, depending on the type of stress applied or the antioxidant used.

This work has shown the complexity of free radical generating models. It is difficult to produce O_2^- in experimental conditions compatible with cellular life. Our results confirm that both the HX–XO and flavin photooxidation systems do not enable SOD-like antioxidants to be studied with the MTT method. Both systems, however, as well as the application of oxidative stress mediated by hydrogen peroxide, are suitable for the study of catalase-like molecules on cultured cells. The action of hydroxyl radical scavengers should be studied in greater detail in the experimental model of cell adhesion and proliferation capacities. The results we obtained with mannitol or DMSO are inconsistent with those of Simon,[24] who used the ^{51}Cr

technique. This author concluded that mannitol protected and that DMSO offered no protection. It should nevertheless be noted that contradictory results were reported by Link,[21] who showed that mannitol did not protect cells from an H_2O_2-mediated stress. However mannitol is reported in the *Merck index* as forming a stable complex with H_2O_2.[26]

These simple and reliable techniques requiring no costly or radioactive equipment are currently being adapted to other cell types, including keratinocytes and endothelial cells. These methodologies should enable the antioxidant capacity of natural or synthetic molecules to be evaluated. Their application to isolated human cells will lead to a better approach to the understanding of the pathophysiological mechanisms of diseases, e.g., cystinosis, cystic fibrosis or Menkes disease.

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