ASCORBOSILANE C - T1

FREE RADICALS

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1 - MEMBRANE PROTECTION

2 - SCAVENGING EFFECT

GENERAL CONCLUSION

1 - CELL MEMBRANE PERMEABILITY AND FREE RADICALS : SILANOLS PROTECTIVE EFFECTS

ABSTRACT

The free radical toxic effect due to their oxidative effect on cells, increases tissue aging process and particularly skin's one.

When peroxidation of membrane lipoproteins occurs, membrane is destroyed, the biological system is damaged, and its maintenance depends on the structural and functionnal integrity of the different membrane lipoproteins.

The way lipidic peroxidation leads to functionnal disturbance is not well known, but it increases membrane permeability.

Cell membrane is particularly affected by free radicals, this is why we studied protective antiradical effects at this level.

INTRODUCTION

In the last few years, an extraordinary interest for radical phenomena has been developed. If these phenomena have been recognized for a long time now, the results of research in this field are relatively recent: discovery of superoxide anions dates back only to 1968, that of superoxide dismutase to 1969. And it is only since 1985 that we have been aware of the existence of free radicals in biological phenomena.

Today, medical and cosmetological research is investigating free radical role in tissue deterioration, alteration of DNA and in the immune system, which play a part in the genesis of several pathological conditions.

If free radicals are activated chemical species, produced in vivo under normal biological conditions, various aggressions and ultraviolet radiation in particular may increase their formation.

Detoxification, enzymatic (glutathione peroxidase, superoxide dismutase) or chemical (vitamins C, A, E) systems naturally protect the organism against free radicals. But when such defense systems are insufficient, these highly reactive molecules can exert their "bad" effects.

Thus, at cell level, peroxidation of polyunsaturated fatty acids in phospholipidic membranes leads to the formation of cytotoxic peroxides which contribute towards inflammatory phenomena and cell death. Moreover, intermediate oxygenated radicals are true activators of carcinogenesis.

At cell level, degradation of the main components of the extra-cellular medium has been observed, which induces modifications in tissue permeability and structure, particularly for skin.

These changes are a contributing factor in skin aging. The use of effective free radical captors is therefore of great interest in cosmetology. It is with this purpose that we studied the efficacy of silanol compounds in reducing free radicals.

To fight free radical phenomenon, several strategies can be considered:

- avoid free radical formation, (sunscreens),
- detoxify formed radical, scavenging effect,
- reduce their bad effects, antioxidant action,
- reinforce natural defence of the organism.

Depending on the activity we search for, we must use an appropriate experimental protocol.

TECHNIQUE I : BIOCHEMICAL APPROACH TO ESTIMATE AN ANTI FREE RADICAL EFFICACY

Free radicals put in a fibroblast culture, can induce cytotoxity, corresponding to cell lysis and an increase in Lactate deshydrogenase (LDH) in the culture medium.

This test consists in producing oxygenated free radicals by enzymatic means. This is perfected by oxydizing acetaldehyde into acetate with xanthine oxydase and submitting cultivated fibroblasts to this radical aggression, with or without silanol derivative. Then Lactate Deshydrogenase activity is measured since this enzyme is a marker for cell membrane rupture. So this test measures membrane resistance submitted to a peroxidative system.

MATERIAL AND METHODS

- Cell Culture :

Human fibroblasts are isolated from fragments of skin from which subcutaneous fatty tissue has been removed. The 1 mm^3 pieces are placed in petri dishes and covered with 1 ml of minimum essential medium (MEM) completed with 10% fetal calf serum (FCS), 200 IU/ml of penicillin, 100 μg/ml of streptomycin and 2 mM of glutamine. Cultures are incubated at 37°C in a humid atmosphere with 5% CO_2.

The fibroblasts grow from the explant ; they can be reseeded after two or three weeks. The cells are removed from the petri dish after a 5 minute exposure to a trypsin solution and collected after centifugation. Fibroblasts, after they are reseeded can only be used for experimentation after a few passages.

Fibroblasts are divided into 2 lots :

- a control batch,
- a treated batch with silanol at 30 mg/l of silicon.

After a 10 day culture, cell populations are suspended after trypsination and subjected to the following treatment :

- Method :

The cell culture medium is subjected to initial centrifugation (2000 rpm/10mn). A pellet (A) and supernatant (B) are retrieved. LDH activity is evaluated in this supernatant, and used as an analytical control showing spontaneous lysis.

Pellet (A) is again suspended in 1 ml of phosphate buffer. In this reconstituted medium, the effects of the oxygenated free radical producing system are studied. After the system has acted, the medium is centrifuged and LDH activity in the supernatant (B) evaluated.

LDH activity is evaluated by a classic UV kinetic method.

RESULTS

	Treatment	% inhibitionor activation
Without oxygenated free radicals	Si 30 mg/l	- 73%+/-20%
With oxygenated free radicals	Si 30 mg/l	- 77%+/-23%

Table 1 : LDH content expressed in % of inhibition or activation compared to the control reference.

DISCUSSION

Results show that Silanol proctects fibroblasts from spontaneous cell lysis even when oxygenated free radicals are added to the culture medium, because LDH with Silanol is much lower than the referenced LDH .

This protection remains among cultures subjected to the radical system.

Free radicals damage cells by the means of two mechanisms : they affect cell membrane in the first place, but also damage vital internal components (such as DNA) when there is no protective system left in the cell .

Also, cell membrane, which is relatively not protected, is the first aim for free radical attack, whereas internal cell structures are better protected particularly because of the joined action of catalases and peroxidases .

The peroxidative system used : HX-XO generates O_2^- and H_2O_2. Cytotoxity depends on H_2O_2 (3). H_2O_2 can be formed by two different ways : XO catalyses a direct formation of both O_2 and H_2O_2. H_2O_2 is also generated by a general spontaneous dismutation of O_2 (4).

Peroxidation of polyunsatured fatty acids from cell membrane is one of the results to free-radical reaction .

In this study, we used a physical technique , Electronic Spin Resonnance (E.S.R) to provide informations on the structure and order of biological membranes (5).

TECHNIQUE II : APPLICATION OF THE SPIN-MARKING METHOD (E.S.R.) TO STUDY THE ANTI FREE RADICAL ACTIVITY

In this study, we used an Electronic Spin Resonance (E.S.R.) technique to obtain information about fibroblast membrane of human skin, with or without treatment with silanols.

MATERIAL AND METHODS

- Cell Culture

The fibroblast culture is realized following the above described protocol.

The cell population is divided into 3 culture batches:

- a control batch and 2 silicon-treated batches with silicon concentrations of 3 and 30 mg/l, respectively. After 10 days, the cells are placed in suspension in a culture medium and processed for E.S.R analysis.

- Spin marking

A 5-doxyl stearic acid solution at 10^{-4} M concentration, which is a spin-marker, is prepared in Dulbecco medium before each experiment.
This solution is kept in a cool place.
The suspended cells are centrifuged and taken up in an unmarked buffer. The cell pellet is then resuspended in a spin-marker solution with a cell density of about 10^6 cells/50μl. After 10 minutes of incubation, the marked cell suspension is transferred to a capillary tube for ESR experimentation.

- E.S.R. measures

The spectra are measured on a BRUKER ER 200 D spectrometer equiped with a double cavity (ER 4105 DR mode TE 104) at a micro-wave frequency of 9.58 GHz.

The corrected order parameter "s" is computed according to HUBELL and McCONNEL's (6) method, with the formula:

$$s = \frac{2T'_{//} - 2T'}{2T_{//} - 2T} \quad 2T_{//} - 2T = 52\ G$$

T'// and T' are anisotropic hyperfine constants experimentally measured on the spectra whereas T// and T are observed during total immobilization in a diamagnetic crystal.

The order parameter shows the degree of liberty of fatty acid chains fluctuating around an average position showing a certain orientation for the magnetic field. The order parameter decreasing even more when the degree of liberty of fatty chains increases, gives information about difference between the ordered structure of crystal, taken as a reference. It enables to obtain an estimation of the existing disorder in the bilayer of phospholipids.

Correlation time gives the average rotation speed of the marker molecules in their environment.It thus measures indirectly the fluidity of this environment, indicating the average of individual mobilities of the chains one to another.

RESULTS

Culture Medium	Order parameter for 5-doxyl stearic acid "s"	% of reduction or increase of the order parameter
Reference	0,70+/-0,06	-
Silanol 3 mg/l Si	0,77+/-0,02	+ 9,1%
Silanol 30 mg/l Si	0,81+/-0,03	+ 15,7%

Table 2 : Measures of the order parameter "s", expressed in % of reduction or increase of the order parameter concerning membrane lipids.

DISCUSSION

The spin marker is, in this study , highly lipophilic, and distributes itself in the whole cell membrane.

Normal fibroblasts treated with Silanols suggest an increase of 2T// and a reduction of 2T . These variations were interpreted as modifications in "fluidity" in the spin marker environment. In a system as complex as a biological membrane, marker's motion is anisotropical and the word "order" fits better. Results show an increase of the order for cell membrane lipids (7).

CONCLUSIONS

Experimental protocols showing free radicals' bad effects are numerous.

Loeper works (1, 2) have shown that silanols have a protective effect for atheromatous phenomena, in which activated oxygen plays a part .

Based on these results, we oriented our research of cosmetic molecules active against free radicals towards silanols.

Cosmetics being based on skin, the experimental protocol we chose was fibroblastic culture with an enzymatic system forming free radicals. Fibroblasts come from human skin.

This model studies free radical attack on whole cells, better than on a specific aim like : DNA, mitochondria...

The external cell membrane is not as well protected as internal constituents which take the benefit of enzymatic activities : catalase and peroxidase (8) . This is why we based our study on cell membrane responses to external attack of the cells. The enzymatic system used to generate free radicals gives external free radicals, which is different from U.V.s which kill cells by formation of intracellular free radicals.

LDH dosage in the fibroblast medium submitted to the system HX-XO, shows silanols ' protective action for cell lysis. This protection corresponds to an increase in fibroblast membrane resistance, when they are cultured in the presence of silanols.

Application of E.S.R technique provides information on the internal structure of cell membrane. When fibroblasts are cultured in the presence of silanols, they increase order in their membrane lipids.

Silanols' anti-free radical activity is not related to a direct scavenging effect, but to an active effect on membrane lipid reorganization, thus making it more resistant to free radical attack.

2 - ASCORBOSILANE C'S SCAVENGING EFFECT

We first have described the anti-radical activity specific to the silanols, but silanols also have an activity peculiar to the molecule to which Silicon is linked. ASCORBOSILANE C provides different anti-radical activities for Silicon and ascorbic acid, this last one providing a direct effect : a scavenging effect.

To prove this scavenging effect, we have used a system hypoxanthine-xanthine oxidase (HX-XO), to prove this scavenging effect, on cultured human fibroblasts.

MATERIAL AND METHODS

Chemical products : XO, xanthine oxidase, grade I (Sigma), MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, HX, hypoxanthine (Sigma)

Cell culture : Normal human fibroblasts are cultured in MEM (Minimum Essential Medium) and 10% FCS (fetal calf serum), and maintained in a 5% CO_2-air atmosphere at 37°C. Cell density is adjusted to 10^6 cells/15 ml of culture medium and incubated during 5 days before the cytotoxicity test. For the study, cells are placed after trypsination in microplates 96-vessels, at a concentration of 20 000 cells per vessel. They grow during two days in 200 μl of MEM + 10% FCS at 37°C in a 5% CO_2 atmosphere.

Evaluation of cell viability : Cell growth and cell survival were quantified by a colorimetric method : the MTT test (Mosmann, 1983). MTT is a tetrazolium salt for which the tetrazolium nucleus breaks down with active mitochondria, by several deshydrogenase enzymes. In these reactions, the pale yellow substrate transforms into a dark blue formazan by living cells, not by dead cells nor by the culture medium. MTT is dissolved in a phosphate buffer (PBS) (0.5 mg/ml at pH 7.4), filtrated to eliminate unsoluble residues which are present, and added to cell vessels (100 μl/vessel). After 4 hours of incubation at 37°C in a 5% CO_2-air atmosphere, 150 μl of acidified isopropanol (HCl, 0.04N) are added to cell vessels and delicately stirred all night to dissolve the blue formed cristals. Readings are perfected with a spectrophotometer (ELISA reading) at 570 nm. Absorbance or optical density (OD) is directly proportional to the number of living cells.

Free radical formation : XO is diluted in PBS and used at 8 mU/ml concentration; HX concentration is 40 μg/ml. For XO and HX, pH is adjusted to 7.4 and 100 μl of each is distributed in vessels after rinsing cells with PBS. After 150 minutes, reaction is stopped by rinsing cells with PBS. The MTT test is then used to determine cell viability. The number of living cells shows toxicity level.

Anti-free radicals : Tested silanols are placed together with the HX-XO system, with a final volume of 100 μl/vessel. Ascorbosilane C's activity of methylsilanetriol ascorbate , (Silanol) is studied at several concentrations : 10, 5, and 1 mg/l, expressed in silicon.

RESULTS

Ascorbosilane C's effect with the HX-XO system was studied. Cell viability is immediately determined after 150 minutes of exposure. Each experience is done using a microplate with 12 vessels for each treatment group.

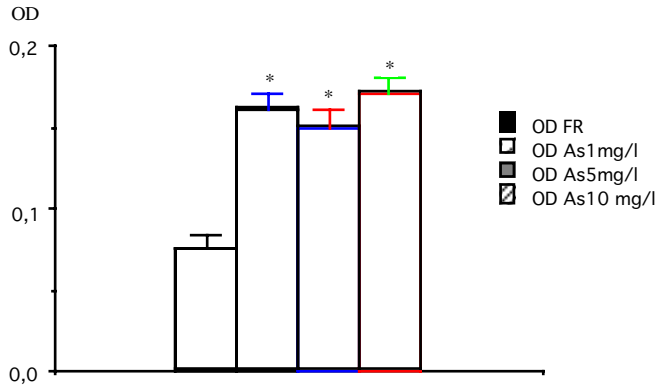

Figure 1 : Relation between optical density for human fibroblasts and concentration of Ascorbosilane C after cell exposure to the HX-XO system.

Values are average +/- SD ; asterisks show a significant difference (variance analysis) compared to the reference (*p<0.05).

FR = reference cells submitted to the system HX-XO
AS10 =cells submitted to the system HX-XO and to Ascorbosilane C at 10 mg/l concentration.
AS5 =cells submitted to the system HX-XO and to Ascorbosilane C at 5 mg/l concentration.
AS1 =cells submitted to the system HX-XO and to Ascorbosilane C at 1 mg/l concentration.

DISCUSSION

To study free radical aggression on skin, we used a model enabling to study cytotoxicity generated by free radicals, on fibroblasts from a human origin. The benefit of this model is to affect the whole fibroblast rather than a specific aim (DNA, microsomes, mitochondria).

This is a rapid and reproducible method, helping to study radical protective agents. With ASCORBOSILANE C, free radical cytotoxicity is very much inhibited.

This reduction can be due to a direct scavenging effect on O_2^- and to a direct inhibition of membrane lipid lipidoperoxidation. Loeper (1,2) has described silanol inhibition on lipidic peroxidation, during a study on the experimental atheroma on rabbits.

GENERAL CONCLUSION

Ascorbosilane C provides two interests towards free radicals.

- As all Silanols, playing a part in cell membrane organization, Ascorbosilane C makes them more resistant to free radical attack :

Membrane protection effect.

- The presence of Ascorbic acid closely linked to the Silanol structure gives another direct intervention on free radicals :

Scavenging effect.

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