Vitamin C and oxidative stress on cultured human keratinocytes

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The aim of this research was to study the hypoxia-induced oxidative stress on cultured human keratinocytes. Control and cultured human keratinocytes were treated with vitamin C, and were then assessed by determining the concentration of malonyl dialdehyde (a final product of lipid peroxidation) existing in the cultured environment. Forty-eight flasks with culture of keratinocytes in confluent layer were divided into four groups: one control and one experimental group treated with vitamin C, and one control and one experimental group without vitamin C. All the cultures were sampled for malonyl dialdehyde assessment immediately after the end of a 30 min period of hypoxia. The statistical analysis of the results showed that vitamin C was not an effective protective agent against oxidative stress caused by hypoxia.

Key Words: *Cultured cells; Free radicals; Human keratinocytes; Oxidative stress; Vitamin C*

Several medical procedures are used to treat the loss of skin depending on the extent of the involved area and the complexity of the case. Secondary healing, excision, primary suture, and total or partial skin patch grafting have all been used. However, in cases of extensive skin loss, none of the aforementioned techniques are possible.

The culture of keratinocytes was a great scientific advance for medical use. A small skin patch can be grown up to 5000 times in three to four weeks (1) providing a considerable amount of epithelial surface. However, its use, in patients who suffered extensive burns, failed to fulfill the medical expectations (2).

Many studies contributed to the development of a culture medium that protects keratinocytes against deleterious conditions (3,4). These advances may be useful in the clinical application of cultured tissue.

The role of free radicals in tissue damage due to oxygen deprivation has been the subject of several studies. Long periods of anoxia cause blood microcirculation to alter, which may result in endothelial edema due to increased vascular permeability,

La vitamine C et le stress oxydatif sur des cultures de kératinocytes humains

La présente recherche visait à étudier le stress oxydatif causé par l'hypoxie sur des cultures de kératinocytes humains. Des kératinocytes témoins et cultivés ont été traités à la vitamine C, puis évalués en établissant la concentration de malonyl dialdéhyde (un produit final de la peroxydation lipidienne) contenue dans l'environnement cultivé. Quarante-huit fioles de culture de kératinocytes en couches confluentes ont été divisées en quatre groupes : un groupe témoin et un groupe expérimental traités à la vitamine C, et un groupe témoin et un groupe expérimental sans vitamine C. Toutes les cultures ont été échantillonnées en vue d'évaluer le malonyl dialdéhyde dès la fin d'une période d'hypoxie de 30 minutes. L'analyse statistique des résultats révèle que la vitamine C n'est pas un agent protecteur efficace contre le stress oxydatif causé par l'hypoxie.

which in turn may block the blood supply leading to tissue necrosis (5). Free radicals combine among themselves in various ways. They are toxic to the cell (6), and may participate in a chain of events resulting in tissue necrosis, unless they are neutralized by a group of substances called antioxidants (7,8).

Because the mechanisms of cellular death involving free radicals are linked to lipid peroxidation (6,9), the activity of free radicals can be assessed in a simple and reliable way: by evaluating the amount of malonyl dialdehyde (MDA), a low weight aldehyde containing three atoms of carbon, which is a stable product of lipid peroxidation (6,10).

Vitamin C acts as a potent antioxidant (11), neutralizing the oxygen toxic reactive specimens and nitrogen reactive specimens (12). Furthermore, vitamin C is an effective antioxidant because after it reacts with a free radical, it becomes the ascorbil free radical, which has a low potential of reduction (13). This ascorbil radical reacts with another identical one producing vitamin C and dehydro ascorbic acid (DHA). Vitamin C can then be regenerated from the reaction of ascorbil radical and DHA (14,15).

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TABLE 1**Experimental and control groups**

Groups	Without vitamin C	With vitamin C
Control	6	6
Experimental (hypoxia)	9	9

An experiment studying the antioxidant action of vitamin C by necrosis flap percentage and free radical production evaluated by MDA was conducted in 1996 (16). It demonstrated that vitamin C significantly shortened the necrosis flap area and resulted in the reduction of lipid peroxidation in the blood and normal skin. However in the skin flap the MDA level was higher than in the control group.

The aim of the present study was to assess the oxidative stress on cultured human keratinocytes subject to hypoxia, with and without the addition of vitamin C, an antioxidant agent, to culture medium. The oxidative activity during oxygen deprivation was assessed by evaluating the level of MDA present in the culture medium.

MATERIALS AND METHODS

Cell culture

Human keratinocytes from skin scraps normally disposed of after surgery were grown according to revised procedures (4) outlined by Green et al (3). They were introduced as approved protocol in our laboratory by Gragnani in 2002 (17).

The keratinocytes were grown to a confluent layer in 75 cm² flasks. The cells were then treated with fresh trypsin, resuspended in culture medium and seeded in 25 cm² flasks previously prepared with 3T3-J2 fibroblasts. Each of these flasks received 25 × 10⁴ keratinocytes in 4 mL of culture medium. All flasks were kept in a humid incubator set at 37°C and with a 5% CO₂ environment. Their content was checked daily for any possible contamination or undesirable growth and the culture medium was replaced as indicated in the protocol.

Vitamin C

The keratinocytes were kept in the aforementioned condition until a confluent layer was attained, after which the supplementation of culture medium with 50 µg/mL of vitamin C (Sigma Chemical Company, USA). This vitamin C supplementation was added 8 h before the experiment commenced. The same volume of vitamin C (50 µg/mL) was added 5 min before the experiment.

Experimental and control groups

After 8 h the experiment commenced. Six flasks were treated with vitamin C, the antioxidant treatment, (experimental) and six without, and were kept on a regular supply of oxygen (control). Of the other 18 flasks, nine were treated with vitamin C and nine were not, and were subjected to 30 min periods of hypoxia (Table 1).

Hypoxia

All the flasks received an air-tight stopper, which was pierced through with two 30 × 7 cm disposable injection needles. To lower the concentration of O₂ inside the flasks, the flasks were put on a shaker and a gaseous mixture containing 95% N₂ and 5% CO₂ was forced through one of the needles for approximately 30 min as proposed by Durão (18). Simultaneously, and for the same period of

Figure 1) Average of the malonyl dialdehyde (MDA) levels of the control group with and without vitamin C (Vit. C) and experimental group with and without vitamin C

time, the open control flasks remained out of the incubator on an identical shaker.

Oxidative stress assessment

Cultivated cell samples were collected from all flasks for MDA assessment. They were stored at 4°C for seven days and then re-stored at -80°C. Oxidative stress assessment was performed by determining MDA concentration in cellular medium using the tiobarbituric acid method (TBA) modified by Percário et al (10), where 1 mL of TBA solution (10 mM TBA in 75 mM potassium phosphate buffer) was added to a 500 µL culture sample, water-bath heated to 95°C for 60 min and left to cool to room temperature or cooled in running water. After cooling, 4 mL of n-butilic alcohol was added and the sample was stirred for homogeneity of the suspended cells, following its centrifugation at 3000 rpm for 15 min. Three mL of the supernatant was collected for spectrographic reading at 535 nm. MDA concentration in ng/mL was determined from two readings.

Statistics

Nonparametric test for k independent samples of Kruskal-Wallis, were complemented when necessary with multiple comparison tests. The P value of 0.05 (5%) was considered as a limit above which the null hypothesis was rejected.

RESULTS

Figure 1 shows the average concentration of MDA for each experimental condition. It shows that MDA levels were consistent with the level of stress to which the various experimental groups were subject. It also shows that vitamin C does not protect cells against oxidative stress and the MDA level in the hypoxia group with vitamin C was higher when compared with the hypoxia group without vitamin C.

DISCUSSION

Experimental models of research in necrosis have developed greatly in the last decades. Anatomical, physiological and cellular studies are important to obtain advances in the knowledge required for daily use in clinical treatment.

Hypoxia is related to many factors that may affect wound healing. Many models of oxygen deprivation have been proposed, but gaseous substitution within the cellular environment is the most used. This deprivation could be reached in an incubator (19-21) or inside flasks (22,18).

Cells damaged due to hypoxia and mechanisms associated with oxidative stress establish lipid peroxidation, which may be evaluated indirectly by MDA assessment.

Vitamin C is not produced by human metabolism, it has low toxicity, it is cheap and it is easy to manipulate. The experimental dose of vitamin C in this study, 50 µg/mL, was chosen based on a review (17,23). The vitamin C showed an active process of intracellular penetration that was maximum at 8 h (24).

Recording MDA levels only at the end of the experiment, 8 h after supplementation with vitamin C for the control and experimental groups and an additional adverse condition of 30 min hypoxia between start and end points of experiment, was adopted because the events created experimentally are

similar to the mechanisms of an ischemic event followed by reperfusion.

The mean values of the concentrations of MDA between the two experimental and two control groups, showed in Figure 1, indicate that hypoxia increased the MDA levels, producing an important, but not significant, oxidative stress. When the two experimental groups were compared, the one with vitamin C supplementation showed an increased MDA level, and it is possible that the vitamin C had a pro-oxidant action in this condition.

Vitamin C research should be continued whether or not it is associated with substances that neutralize metals in the culture medium. The evaluation of vitamin C penetration associated with other antioxidants is required to determine its function. Any study to determine the protective effect of vitamin C against oxidative stress due to hypoxia should be encouraged. The determination of the most effective antioxidant agents has great practical application in plastic surgery where hypoxia plays a major role in unsuccessful outcomes.

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