

Keratinocyte growth factor protected cultured human keratinocytes exposed to oxidative stress¹

Fator de crescimento de queratinócitos protegeu queratinócitos humanos cultivados expostos ao estresse oxidativo

Alfredo Gragnani^I, Marina Bertelli Rossi^{II}, Janne Cely Medeiros Albuquerque^{III}, Gabriela Soares Silva Brito^{IV}, Antonio Carlos Aloise^V, Lydia Masako Ferreira^{VI}

^I MD, PhD, Affiliate Professor, Plastic Surgery Division, UNIFESP, Sao Paulo, Brazil.

^{II} Nursing Graduate student, UNIFESP, Sao Paulo, Brazil.

^{III} Student of Extension Course in Plastic Surgery, UNIFESP, Sao Paulo, Brazil

^{IV} Fellow Master Degree, Plastic Surgery Post-graduation Program, UNIFESP, Sao Paulo, Brazil.

^V Fellow PhD Degree, Plastic Surgery Post-graduation Program, UNIFESP, Sao Paulo, Brazil.

^{VI} Chairwoman, Full Professor, Plastic Surgery Division and Head of Surgery Department, UNIFESP, Sao Paulo, Brazil.

ABSTRACT

Purpose: To evaluate effects of oxidative stress and supplementation of keratinocyte growth factor (KGF) on cultivated human keratinocytes. **Methods:** Oxidative stress was produced through addition of hydrogen peroxide (H₂O₂) to the culture medium. Cultivated human keratinocytes were divided in 4 groups: Group control (G C), Group KGF (G KGF), Group H₂O₂ (G H₂O₂), Group H₂O₂ and KGF (G H₂O₂-KGF). Each experiment was accomplished with the same lineage cultivated keratinocytes, in triplicate. Cell viability was evaluated by trypan blue exclusion assay. **Results:** The results showed that the culture medium supplemented with KGF presented a small rate of cell viability when compared to cells only in culture medium (p<0,001). It demonstrated that only the growth factor does not have protector effects for cells in vitro. However, in front of the oxidative stress produced by addition of hydrogen peroxide to the medium, KGF showed a beneficial effect, protecting cells when compared to the group that suffered hydrogen peroxide action but had not been exposed to KGF (p<0,001). **Conclusion:** KGF determined protection to the primary human keratinocytes exposed to oxidative stress.

Key words: Keratinocytes. Cell Survival. Oxidative Stress.

RESUMO

Objetivo: Avaliar os efeitos do estresse oxidativo e da suplementação do fator de crescimento de queratinócitos (KGF) em queratinócitos humanos cultivados. **Métodos:** O estresse oxidativo foi produzido através da adição de peróxido de hidrogênio (H₂O₂) ao meio de cultura. Os queratinócitos humanos cultivados foram divididos em quatro grupos: grupo controle (G C), grupo KGF (G KGF), grupo H₂O₂ (G H₂O₂), grupo H₂O₂ e KGF (G H₂O₂-KGF). Cada experimento foi realizado com a mesma linhagem celular, em triplicata. A viabilidade celular foi avaliada pelo ensaio da exclusão do azul de tripan. **Resultados:** Os resultados mostraram que o meio de cultura suplementado com o KGF apresentou menor taxa de viabilidade celular quando comparado às células do grupo controle (p<0,001). Isso mostra que somente o fator de crescimento de queratinócitos não apresentou efeito protetor às células em cultura. Entretanto, frente ao estresse oxidativo produzido pela adição do peróxido de hidrogênio ao meio de cultura, o KGF mostrou efeito benéfico, protegendo as células quando comparado ao grupo que sofreu a ação do estresse oxidativo, mas que não foi exposta ao KGF (p<0,001). **Conclusão:** O KGF determinou a proteção aos queratinócitos humanos primários cultivados expostos ao estresse oxidativo.

Descritores: Ceratinócitos. Sobrevivência Celular. Estresse Oxidativo.

¹Research performed at the Laboratory of Culture of Cell, Plastic Surgery Division, Surgery Department, Federal University of Sao Paulo (UNIFESP), Brazil.

Introduction

The essential function of human skin is organism protection. The cutaneous barrier loss causes serious failures in organism protection system either on mechanical or immunological level.

The keratinocytes form all layers of the epidermis and their metabolism become progressively smaller as far from the basal layer. They do not exist in horny layer and constitute a double layer

structure, composed of keratin involved by an envelope, which previously was the cellular membrane, and lipids, both responsible for human survival through maintaining liquids and proteins in the internal environment, and impeding the invasion of external microorganisms. The horny layer of the epidermis is also responsible for resisting to physical and chemical aggressions, and the permeability of skin to water.

The central objective of burn patient's treatment is to recover of cutaneous barrier. Major burn patients hardly survive for a long time, because the regeneration time of the skin takes too long to be completed and the infection rate is high. In addition of small donor area in major burns, the use of definitive wound coverage with cultivated keratinocytes makes possible patient survival¹.

The use of simple sheet of cultivated keratinocytes on the wounds shows the disadvantage of low graft integration rate and a consequent period of prolonged rest in order to conclude the wound healing process. After the end of this process, the possibility of emergence of new lesions due to covering fragility still remains².

In the treatment of the skin substantial losses, as in major burns, the patient who is already compromised by cutaneous barrier loss still submitted to several surgical procedures for devitalized tissue excision before submitted to skin definitive coverage through autografting or keratinocytes culture graft.

The autogenous graft of skin could be full or partial thickness, and the first is only applied to small defects and in specific situations. It has little usefulness in burn patients with great committed extension. The grafts of partial thickness could be used as a sheet or could be meshed to covering the lesions and, where the technology exists, also for sheets of cultivated human keratinocytes.

In these types of treatment they are present countless systemic or local aggressive factors. In relation to receptor, during the clinical application of autografts, when donor areas exist or it is insufficient for the utilization of keratinocytes culture, several factors that interfere with a good adherence and integration of these grafts may be found. Local or systemic alterations generated or not generated by the cutaneous integrity loss, harm the arrival of oxygen and nutritious to wounds³.

The offer of those elements is fundamental for the coverage success, mainly during the plasmatic liquid absorption phase of the skin graft. Among those factors, hypoxia is one of the main unsuccessful factors and causes an increase of the morbidity and mortality rate of these patients.

The keratinocytes growth factor (KGF) is a growth factor produced by dermal fibroblasts which possess receptors in epithelial cells. The main characteristic of KGF is to stimulate keratinocytes proliferation and it has been used in experimental models of cutaneous lesions and it is in the beginning of clinical studies.

In vitro, the aggressions caused by cellular oxygen privation have been observed and it can besides to make unfeasible the process of graft integration. However, there are no studies in the literature about the oxidative stress effects in human keratinocytes culture supplemented with KGF as probable protection for such aggression.

The aim of this study was to evaluate the effects of oxidative stress and supplementation of KGF on cultured human keratinocytes.

Methods

Culture of primary human keratinocytes

The culture protocol defined by Green, Kehinde, Thomas⁴ and Green⁵, was applied in the Laboratory of Cell Culture UNIFESP's Plastic Surgery Division by Gragnani, Morgan, Ferreira^{6,7}.

This present study was analyzed by UNIFESP/EPM's Ethics Committee and approved under number 0395/07. After obtaining patient consent term, fragments of skin discarded from plastic surgery were used for obtaining the isolation of primary human keratinocytes.

The primary culture of keratinocytes started with a suspension of isolated cells derived from a full thickness fragment of patient's skin, which was cut up and treated with trypsin in order to disaggregate the cells.

The keratinocyte culture medium was a 3:1 mixture of 750 mL Dulbecco's Modified Eagle's Medium (DMEM) (high glucose [4.5 g/L], L-glutamine [584 mg/L], and sodium pyruvate [110 mg/L]) and 250 mL Ham's F-12 medium, totaling a volume of 1 L. This was supplemented with 10% fetal bovine serum (FBS) and 24 mg of freshly prepared adenine (6-aminopurine hydrochloride) diluted in 20 mL DMEM/Ham's F-12 mixture, resulting in a final concentration of 1.8×10^{-4} mol/L. 1 ml of 10^{-10} mol/L cholera toxin (*Vibrio cholerae*, Type Inaba 569 B), 2 mL of penicillin/streptomycin (100 UI/mL, 100 µg/mL), 2 mL of 0.4 µg/mL hydrocortisone, 1 mL of 2×10^{-9} mol/L transferrin/triiodo-L-thyronine and 1.3 mL of 5 µg/mL insulin (porcine) in a final concentration of 5 µg/mL. The pH was adjusted to approximately 7.2; the medium was sterilized with a 0.22-µm filter, kept in a refrigerator at 4°C, and produced a red color.

Normal human keratinocytes were derived of child's prepuce. Fragments of 1 cm² of total skin discarded in the surgical center were obtained in the morning when the cell isolation was performed.

The initial step in a laminar flow hood was to wash the skin fragment in tubes containing phosphate buffered saline (PBS) and then fragmented into small pieces in 0.05% trypsin. The fragments were placed in a preheated shake flask containing 6ml of trypsin and 6 ml of versene in order to determine separation and primary isolation of keratinocytes. After 30 minutes, the supernatant solution was removed. The solution was centrifuged and the cells were resuspended in 3ml of keratinocytes culture medium and seeded on flasks with 3T3 J2, fibroblasts pre-treated on it. The flasks were placed in humid incubator at 37°C and 5% CO₂, and the medium was changed every two days, until the pre-confluency status was obtained, and the cells were used in the experiments.

KGF

After isolation of primary human keratinocytes, KGF was added in the respective groups for 30 minutes and all of tubes with cells were placed in humid incubator at 37°C and 5% CO₂.

Oxidative stress

The oxidative stress was produced through the addition of 50µM of hydrogen peroxide (H₂O₂) to respective tubes, after

the KGF supplementation in appropriate groups. All of tubes with cells were placed in humid incubator at 37°C and 5% CO₂ for 5 minutes, and after this they were evaluated.

Experimental model

Cultivated keratinocytes were divided in 4 groups: Group control (G C); Group KGF (G KGF) ; Group H₂O₂ (G H₂O₂) and Group KGF + H₂O₂ (G KGF H₂O₂).

From the same isolation, same cell lineage, cells were divided in four groups with 250,000 cells in each experiment tube. A group control containing cells with culture medium was not submitted to intervention, but following for the same time and storage place of other groups.

One second group contained cells with culture medium supplemented with KGF. A third group contained the same number of cells with culture medium supplemented with H₂O₂. And a last group contained cells with culture medium and supplemented with KGF and H₂O₂.

Each experiment was carried out in triplicate with cultivated keratinocytes of the same lineage.

Trypan blue assay

Soon after cells were centrifuged at 800 rpm for 6 minutes, the pellet of cells was resuspended in 2 ml of culture medium and later counted in a Neubauer Chamber with Trypan Blue dye in order to define cellular viability.

Statistical method

The variance analysis of double entrance was accomplished for multiple comparisons among continuous variables, and post-Bonferroni test for the comparison of paired continuous variables. The level of statistical significance was fixed in 0.01.

Results

Of 250,000 cells initially placed in the tubes, we obtained the average of 163,800 cells in group control; 61,880 in group where KGF was added; 30,000 in group of cells exposed to H₂O₂; and finally 93,130 in group which suffered oxidative stress and exposed to KGF (Table 1). This result presented statistic difference with significance among all of the groups.

The number of viable cells in each group is shown below (Figure1).

TABLE 1 - Experiment groups presented average of viable primary human keratinocytes after hypoxia through H₂O₂ and the use of KGF, standard error and n=3

Groups	Average	Standard error	n
Control	163,800	7,500	3
KGF	61,880	4,375	3
H₂O₂	30,000	3,750	3
H₂O₂ + KGF	93,130	4,375	3

Two-way ANOVA

Bonferroni post-teste

Control vs KGF P <0.001

Control vs H₂O₂ P <0.001

Control vs KGF+H₂O₂ P <0.001

KGF vs H₂O₂ P <0.01

KGF vs KGF+H₂O₂ P <0.01

H₂O₂ vs KGF+H₂O₂ P <0.001

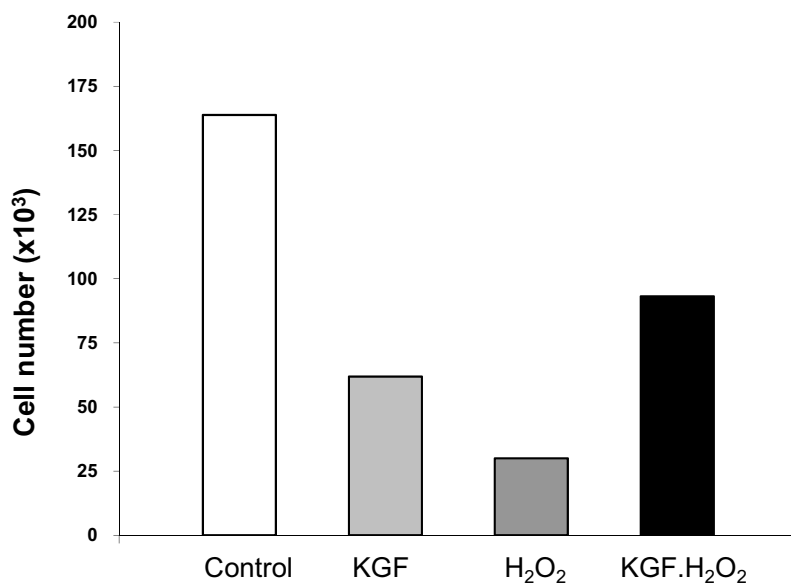


FIGURE 1 - Experiment groups: number of viable keratinocytes after hypoxia through H₂O₂ and KGF exposition

Discussion

More than other tissues, the human skin is exposed to countless external stresses and generates reactive oxygen species (ROS), which in cooperation with endogenous oxygen radicals, cause keratinocyte alterations and partly contribute to photocarcinogenesis and cutaneous aging⁸.

During the past four decades, the involvement of ROS such as hydrogen peroxide, superoxide and hydroxyl radical, has been demonstrated in several clinical conditions⁹. ROS are synthesized inside the mitochondria during the energy metabolism and their overproduction is associated to cardiovascular diseases, inflammatory reactions and degenerative disorders related to the aging process¹⁰.

In normal metabolism, ROS are inactivated by natural antioxidants such as glutathione, superoxide dismutase and the catalase¹¹. When these substances are not capable to neutralize ROS or to inhibit their production, an unbalance among pro-oxidants and antioxidants occurs. This situation is known as oxidative stress.

Some conditions such as the acute or chronic exposition to UV radiation, dehydration, mechanical lesion, changes in the environment temperature or endogenous insults as inflammation and hypoxia can produce oxidative stress in cells¹². An aggression example that gathers several conditions implicated in oxidative stress is skin burn, and depending on the extension and the proportion of tissue loss, may lead to unacceptable consequences in a hemodynamical and functional context.

Graft of partial autologous skin is the "gold standard" for the definitive coverage of burn wounds. However, in some major burn patient, the availability of donor area is limited in the presence of extensive burns¹³.

Keratinocytes play a fundamental role in this regeneration and assume main responsibility for the formation of all epidermis layers. Among others facts, keratinocytes cultivation in laboratory carry out a primary importance to the investigation of oxidative stress-induced cytotoxicity, disturbances produced in mitochondrias and alterations in the intracellular antioxidant potential¹⁴⁻¹⁶.

Fortunately, a considerable progress was reached in human keratinocytes culture and nowadays it is possible to obtain large amounts of cultivated epithelium from a small skin biopsy within 3-4 weeks after the first operative action of burn patient¹³.

Since 1970s, with the Rheinwald and Green's paper, serial cultivation of human keratinocytes became possible. Ever since, several authors have proposed several techniques of keratinocytes isolation in order to optimize the established protocols of cultivation¹⁷⁻²⁰. The methodology of human keratinocytes culture employed in this present work was revised and appropriated by Gragnani, Morgan and Ferreira in our laboratory^{6,7}.

The hydrogen peroxide was chosen to induce oxidative stress in keratinocytes, because studies with primary cultures of undifferentiated keratinocytes have demonstrated that exposition to hydrogen peroxide induced cellular death through a mitochondria failure. Recent evidences suggest a dependent susceptibility

in the differentiation for apoptosis in cells exposed to H₂O₂⁸. The hydrogen peroxide is a ROS formed in larger amount by the attempt of exogenous agents' desintoxication and easily penetrates through cellular membrane to biomolecular damage²¹. The action of hydrogen peroxide is due to the attack to lipid membrane, ADN and other cellular components produced by peroxide's toxic free radicals

Several growth factors break out different cellular answers, such as proliferation, migration and alteration of cellular cycle. Among the growth factors, keratinocytes growth factor (KGF) is a potent mitotic factor of epithelial cells²². KGF is a growth factor produced by fibroblasts and acts in keratinocytes, because KGF receptors is present on these cells, which is one of the communication signs between these two fundamental cells involved in wound recovery of the skin. Therefore, the keratinocytes of epidermis are stimulated by KGF action.

KGF presented an effect of acceleration on the epithelization of partial and total wounds in different experimental animal models, which KGF, when applied in wounds of diabetic mice, was capable to improve the epithelization of these lesions²³.

The present study showed that the primary cultured human keratinocytes supplemented with KGF, separately, presented small cell viability rate, when compared to the control group. It revealed that the factor separately does not possess protective effect to cells in vitro. However, in front of oxidative stress produced by addition of hydrogen peroxide into the medium, KGF showed a beneficial effect, protecting the cells and reducing the apoptosis when compared to the group that suffered action of hydrogen peroxide and had not been exposed to KGF.

Studies support the hypothesis of cellular oxidative stress being a critical stage of burn wounds, and they also suggest that antioxidant strategies that determine inhibition or degradation of free radicals; may stimulate an organic protection for patients who suffered burns, and this issue should be studied more properly to define strategies for the burn treatment²⁴.

Conclusion

KGF determined protection to primary cultured human keratinocytes exposed to oxidative stress.

References

1. Gallico III GG, O'Connor NE, Compton CC, Kehinde THE, Green H. Permanent coverage of large burn wounds with autologous cultivated human epithelium. *New Engl J Med*. 1984;311(7):448-51.
2. Sheridan RL, Tompkins ID-Cultivated autologous epithelium in patients with burns of ninety percent or of the body surface lives. *J Trauma*. 1995;38(1):48-50.
3. Compton CC. Wound healing potential of cultivated epithelium. In: *Inovativ Technologies and Wound Healing: The Clinical and Research Symposium*. 1992;5(2):97-111.
4. Green H, Kehinde THE, Thomas J. Growth of cultivated human epidermal cells into multiple epithelia suitable goes grafting. *Proc Natl Acad Sci USES*. 1979;76:5665-9.
5. Green H. Cultured cells for the treatment of disease. *Sci Am*. 1991;265(5):96-102.

6. Gragnani A, Morgan JR, Ferreira LM. Differentiation and barrier formation of the cultivated composite skin graft. *J Burn Care Rehabil.* 2002;3(2):126-31.
7. Gragnani A, Morgan JR, Ferreira LM. Experimental model of cultivated skin graft. *Acta Cir Bras.* 2004;19:(supl.)4-10.
8. Zuliani T, Denis V, Noblesse AND, Schnebert S, Andre P, Dumas M, Ratinaud MH. Hydrogen peroxide-induced cell death in normal human keratinocytes is differentiation dependent. *Free Radic Biol Med.* 2005;38(3):307-16.
9. Chan SS, Monteiro HP, Deucher GP, Abud RL, Abuchalla D, Junqueira VB. Functional activity of blood polymorphonuclear leukocytes the antioxidant stress biomarker in human subjects. *Free Radic Biol Med.* 1998;24(9):1411-8.
10. Kehrer JP. Free radicals the mediators of tissue injury and disease. *Crit Rev Toxicol.* 1993;23:21-48.
11. Splitz DR, Adams DT, Sherman CM, Roberts RJ. Mechanisms of cellular resistance to hydrogen peroxide, hyperoxia and 4-hydroxy-2-nonenal toxicity; the significance of increased catalase activity in H₂O₂ resistant fibroblasts. *Arch Biochem Biophys.* 1992;292:221-7.
12. Diker-Cohen T, Koren R, Ravid A. Programmed cell death of stressed keratinocytes and its inhibition by vitamin D: The roles of death and survival signaling pathways. *Apoptosis.* 2006;11(4):519-34.
13. Atiyeh BS, Costagliola M. Cultivated epithelial autograft (CEA) in burn treatment: three decades later. *Burns.* 2007;33(4):405-13.
14. Takeyama N, Miki S, Hirakawa THE, Tanaka T. Roles of the mitochondrial permeability transition and cytochrome C release in hydrogen peroxide-induced apoptosis. *Exp Cell Res.* 2002;274:16-24.
15. Berendji D, Kolb-Bachofen V, Meyer KL, Kroncke KD. Influence of nitric oxide on the intracellular reduced glutathione pool: different cellular capacities and strategies to encounter nitric oxide mediated stress. *Free Radic Biol Med.* 1999;27:773-80.
16. Gardner THE, Xu FH, Fady C, Sarafian T, You YP. Evidence against the hypothesis that BCL-2 inhibits apoptosis through its antioxidant effect. *Cell Death Differ.* 1997;4:487-96.
17. Rheinwald JG, Green H. Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells. *Cell.* 1975;6:331-43.
18. Hirel B, Chesne C, Pailheret JP, Guillouze A. Expression of differentiation markers in human adult keratinocytes cultivated in submerged conditions. *In Vitro Cell Dev Biol Anim.* 1994;30A(6):372-8.
19. Marcelo CL, Tong PS. Epidermal keratinocyte growth: changes in protein composition and synthesis of keratins in differentiating cultures. *J Invest Dermatol.* 1983;80(1):37-44.
20. Boyce ST, Foreman TJ, Furmanski P, Hansbrough JF. Absence of tumorigenicity in athymic mice by normal human epidermal keratinocytes after culture in serum-free medium. *Cancer Lett.* 1992;62(2):141-7.
21. Kiruthiga PV, Shafreen RB, Pandian SK, Oved KP. Silymarin protection against major reactive oxygen species released by environmental toxins: exogenous H₂O₂ exposure in erythrocytes. *Basic Clin Pharmacol Toxicol.* 2007;100(6):414-9.
22. Finch PW, Rubin JS, Miki T, Ron D, Aarson SA. Human KGF is FGF-related with properties of a paracrine effector of epithelial cell growth. *Science.* 1989;245:752-5.
23. Werner S, Peter KG, Longaker MT, Fuller-Pace F, Banda MJ, Williams LT. Large induction of keratinocyte growth factor expression in the dermis during wound healing. *Proc Natl Acad Sci USA.* 1992;89:6896-900.
24. Horton JW. Free radicals and lipid peroxidation mediated injury in burn trauma: the roles of antioxidant therapy. *Toxicology.* 2003;189(1-2):75-88. Review.

Acknowledgements

The authors are grateful to undergraduate research scholarship (PIBIC), National Board of Technological and Scientific Development (CNPq).

Conflict of interest: none
Financial source: none

Correspondence:

Alfredo Gragnani
Department of Surgery / Plastic Surgery Division
Federal University of Sao Paulo
Rua Napoleao de Barros, 715/4o andar
04042-002 Sao Paulo - SP Brazil
Phone: (55 11)5576-4118
Fax: (55 11)5571-6579
gragnani.dcir@epm.br

Received: August 26, 2009
Review: October 29, 2009
Accepted: November 25, 2009

How to cite this article

Gragnani A, Rossi MB, Albuquerque JCM, Brito GSS, Aloise AC, Ferreira LM. Kgf protected cultured human keratinocytes exposed to oxidative stress. *Acta Cir Bras.* [serial on the Internet] 2010 Jan-Feb;25(1). Available from URL: <http://www.scielo.br/acb>