ACCELERATED WOUND HEALING OF ORAL SOFT TISSUES AND ANGIOGENIC EFFECT INDUCED BY A POOL OF AMINOACIDS COMBINED TO SODIUM HYALURONATE (AMINOGAM®)

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In this study we investigated the property of a new medical substance, in the form of a gel compound containing four aminoacids (glycine, leucine, proline, lysine) and sodium hyaluronate (AMINOGAM®), to accelerate the wound healing process of the soft oral tissues and to promote angiogenesis *in vivo* in the vascular proliferation in chick embryo chorioallantoic membrane (CAM) assay. Furthermore, we investigated the capacity of AMINOGAM® to induce the expression of an angiogenic cytokine, namely vascular endothelial growth factor (VEGF) in human fibroblasts *in vitro.* Results showed that AMINOGAM® promoted wound healing in post-surgical wounds (after teeth extraction, oral laser surgery with secondary healing without direct suture of the surgical wound, and after dental implant insertion). Stimulated angiogenesis *in vivo* in the CAM assay and the response was similar to that obtained with vascular endothelial growth factor, a well-known angiogenic cytokine, tested in the same assay, and confirmed by clinical outcomes, which showed reduction of the healing time of oral soft tissues after three different kinds of surgery and also the absence of post-operative infections.

Wound healing is a specific biological process related to the general phenomenon of growth and tissue regeneration. It is characterized by the formation of a granulation tissue consisting of inflammatory cells, newly formed blood vessels and fibroblasts embedded in a loose collageneous extracellular matrix. Re-epithelization, angiogenesis and matrix deposition are critical events controlling this process (1).

Angiogenesis is confined to the wound site and plays a pivotal role for successful wound healing (2). Indeed, re-vascularization is required to furnish the new tissue and to dispose of the waste products of metabolism. Angiogenesis occurs as a highly regulated process which is rapidly stimulated after injury and ceases when wound healing is complete. Wound angiogenesis is believed to be initiated by the early release of preformed growth factors, such as fibroblast growth factor-2 (FGF-2) and vascular endothelial growth factor (VEGF) (3-4).

Wound management after surgery implies obtaining healing in the shortest time, with minimal pain, discomfort, and scarring for the patient, leading to the wound closure with a flexible and

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fine scar with high tensile strength. Several factors could impede tissue repair and regeneration such as hypoxia, infection, tumors, metabolic disorders such as diabetes mellitus, the presence of debris and necrotic tissue, whereas certain types of medications and a diet deficient in protein, vitamins, or minerals could delay this process. It is generally accepted that a high availability of aminoacids is necessary in wound repair due to an increased metabolic activity.

Hyaluronic acid is also involved in wound healing. It is a glycosaminoglycan composed of repeating disaccharide units on D-glucoronate and N-acethylglucosamine and is one of the most abundant constituents of the extracellular matrix. Hyaluronic acid is involved in a number of developmental processes and has been shown to promote cell proliferation, differentiation and motility.

Hyaluronic acid is naturally biocompatible, biodegradable and lacks immunogenicity (5). Hyaluronic acid-modified liposomes as bioadhesive carriers for delivering growth factors to wound sites have been studied and reported (6) and more recently, hyaluronic acid demonstrated effective for managing acute wounds, particularly in terms of its safety and efficacy (7).

Traditional wound healing agents include topical liquid and semi-liquid formulations as well as dry traditional dressings. In this study we investigated the property of a new medical substance, in form of a gel compound containing four aminoacids (glycine, leucine, proline, lysine) and sodium hyaluronate (AMINOGAM®), to accelerate the wound healing process of the soft oral tissues and to promote angiogenesis in the vascular proliferation in chick embryo chorioallantoic membrane (CAM), a well established *in vivo* assay for angiogenesis and anti-angiogenesis (8). Furthermore, we investigated the capacity of AMINOGAM® to induce the expression of VEGF in human fibroblasts *in vitro*.

MATERIALS AND METHODS

Clinical study

120 patients (60 for the experimental group and 60 for the control group) were selected. The 60 patients of the experimental group were divided into three subgroups of 20 patients who had undergone three different surgical procedures: subgroup A, underwent dental extraction of molars on both side of the jaw and were treated with AMINOGAM® (three applications/die) upon the extraction sites exclusively on one side; subgroup B, received endosseous dental implants with flap elevations and were treated with AMINOGAM® (three applications/die) until complete healing of the surgical incision; subgroup C received diode laser treatment of benign neoplasms of the oral mucosa without direct suture of the surgical margins (diameter of the soft tissue exposed approximately was 20 mm) and were treated with AMINOGAM® (three applications/die) until secondary healing. For experimental subgroups B and C, excluding subgroup A who had internal control of extraction sites with the opposite sites, an equivalent number of patients were selected as control subgroup. To compare the healing times between the control subgroups and the experimental subgroups, in the former the normal healing times of soft tissues for each surgical procedure were established as follows: for subgroup A, 15 days with complete closure of the socket and 30 days for keratinization of the gingiva; for subgroup B, 12 days with full thickness closure of the edges of the elevated flaps; for subgroup C, 21 days with complete reconstruction of the covering mucosa and keratinization.

In vivo study

Fifty fertilized White Leghorn chicken eggs were incubated at 37°C at constant humidity. On day 3 of incubation a square window was opened in the egg shell after removal of 2-3 ml of albumin so as to detach the developing CAM from the shell. The window was sealed with glass and the eggs were returned to the incubator. At day 8 of incubation, gelatin sponges (Gelfoam, Upjohn Company, Kalamazoo, MI) were cut to 1 mm^3 pieces and placed on top of a growing CAM under sterile conditions. AMINOGAM® in phosphate buffered saline (PBS) was then adsorbed on to the sponges. Sponges containing PBS alone or 200 ng of VEGF (R & D Systems, Abington, UK) were used, respectively, as negative and positive controls. On day 12, the CAM were photographed using a stereomicroscope (SZ-CTV Olympus Optical Co, Rozzano, Italy) connected to a digital camera. Blood vessels entering the sponges within the focal plane of the CAM were counted by two observers in a double-blind fashion at a magnification of x 50, and means values ± 1 standard deviation (SD) were determined. The significance of differences between experimental and control CAM counts was determined by Student's *t* test for unpaired data.

Immunohistochemistry

Two murine monoclonal antibodies (MAb) against the endothelial cell marker CD31 (MAb 1A10, Dako Cytomation, Glostrup, Denmark) and against the

macrophage marker CD68 (NCL-CD68-KP1, Novocastra Laboratories Ltd, Newcastle, UK) were used. Briefly, some bioptic specimens obtained from experimental and control subgroup A were collected on 3-amino-propyl-triethoxysilane coated slides, deparaffinized by the xylene-ethanol sequence, rehydrated in a graded ethanol scale and in Tris-buffered saline (TBS, pH 7.6), and incubated overnight at 4°C with the MAb 1A10 (1: 25 in TBS) and with the Mab KP1 (1:200 in TBS), after prior antigen retrieval by heating the sections in a pressure cooker in 1mmol/l EDTA buffer, pH 8.0 for 1.3 min. For CD31 immunostaining, the sections were incubated with biotinylated IgG and then with peroxidase-conjugated streptavidin (LSAB2, DakoCytomation). The colour was developed by diaminobenzidine. The immunodetection of CD68 was performed with alkaline phosphatase anti-alkaline phosphatase (APAAP, DakoCytomation) and fast red as chromogen, followed by hematoxylin counterstaining. Negative controls included an unrelated monoclonal IgG1 produced by the P3X63/Ag8 mouse secretory myeloma replacing the antibody, for the MAb against CD31 (9) and preincubation with a 10-fold excess of specific blocking peptide (Santa Cruz) for the antibody against CD68.

Microvessel density and CD68 positive cells counting

These were simultaneously assessed without knowledge of the final pathological diagnosis by two investigators with a double-headed light microscope (Axioplan II, Zeiss, Oberkochen, Germany). Four to six 200X fields covering almost the whole of each of three sections per sample were examined with a 144-intersection point square reticulum (0.78 mm^2) inserted in the eyepiece. Care was taken to select microvessels, i.e. capillaries and small venules, from all the CD31-stained vessels. They were identified as transversally sectioned tubes with a single layer of endothelial cells, with or without a thin basement membrane. Each assessment was agreed upon in turn. Microvessels were counted with a planimetric point-count method with slight modifications to restrict counting to transversally cut microvessels occupying the reticulum intersection points (10). As the microvessel diameter was smaller than the distance between adjacent points, only one transversally sectioned microvessel could occupy a given point. Microvessels transversally sectioned outside the points and those longitudinally or tangentially sectioned were omitted. Therefore, it was sufficiently certain that a given microvessel was counted only once, even in the presence of several of its section planes. As almost the entire section was analysed per sample, and as transversally sectioned microvessels hit the intersection points randomly, the method allowed objective counts. Cells stained with anti-CD68 antibody was counted on 4-6 fields covering the whole of each of three sections adjacent to those stained for microvessels and mean ± 1 SD and were determined for each section. The statistical significance of differences between experimental and control counts was determined by Student's *t* test for unpaired data.

VEGF mRNA expression in human fibroblasts

Human foetal lung fibroblasts (MRC-5) were cultured in MEM (Minimum Essential Medium with Earle's salts; Euroclone, Ltd.) supplemented with 10% foetal calf serum, 100 μg /ml penicillin, 250 μg /ml streptomycin and 2 mmol L-glutamine, at 37°C in a humidified atmosphere of 5% CO_2. MRC-5 were utilized between the 26^{th} and 28^{th} passages. Cells ($5x10^5$) were plated into 25 cm^2 flasks for three days. Then medium was replaced with a fresh one. A solution containing 0.125 % aminoacids (glycine, leucine, proline, lysine) and 0.09 % sodium hyaluronate, at the same ratio of AMINOGAM®, in H_2Od was freshly made. This solution was added into the medium at the ratio 1:10 (v/v; final volume 6 ml). Three and four days after starting the assay, cells were harvested, washed twice in PBS and counted. Total RNA was isolated from $5x10^5$ cells by TRIzol reagent (Invitrogen), and spectrophotometrically measured. 2 ng of total RNA primed with oligo (dT), incubated at 42°C for 45 min, were used to synthesize c-DNA by SuperScript III First-strand Synthesis System for RT-PCR (Invitrogen, USA). Reverse transcription was terminated by heating at 70°C for 10 min and 1 μl of c-DNA was used as the template for PCR. Primers to evaluate VEGF expression were: For: 5'- TGTCTTGCTCTATCTTTCTT-3' Rev 5'- CTTGCCTTGCTGCTCTACCT-3', (amplification product of 383 bp). GAPDH was used as housekeeping gene: For: 5'-TTGGTATCGTGGAAGGACTCA-3'; Rev: 5'-TGTCATCATATTTGGCAGGTTT-3' (amplification product of 269 bp). The PCR reaction was done at 94°C for 15 sec, 60°C for 1 min, and 72°C for 1 min for 40 cycles for VEGF amplification, 94°C for 15 sec, 55°C for 30 sec, and 68°C for 1 min for 30 cycles for GAPDH amplification. The PCR products were analyzed on 1.5 % agarose gel and stained by ethidium bromide.

RESULTS

Clinical study

All clinical results are summarized in Table 1.

In the patients of subgroup A, who underwent dental extraction of molars on both side of the jaw and were treated with AMINOGAM® (three applications/die) upon the extraction sites exclusively on one side, a reduction of the elapsed

Table 1. *A GROUP: 20 extraction sites treated with AMINOGAM® and 20 controls (not users) with Standard Deviation (SD)*

	Sites	Suture	Suture Removal (median value)	Healing (median value)	Complications
20 study cases	**Molars**	**yes**	**6.9 days (SD 0.788)**	**11.5 days (SD 1.069)**	**no**
20 control cases	**Molars**	**yes**	**7.6 days (SD 0.502)**	**14.4 days (SD 1.486)**	**25% of case (delayed healing, post-extractive sicca alveolitis, infections)**
P			**0.007**	**< 0.001**	**n.s.**

B GROUP: 20 patients who received endosseous implants of 4.1 mm in diameter by full thickness flap elevation with suture, using AMINOGAM® and 20 controls (not users) with Standard Deviation (SD)

	Suture Removal (median value)	Gingival Healing (median value)	Complications
20 study cases	**6.9 days (SD 0.718)**	**8.4 days (SD 0.753)**	**no**
20 control cases	**10.5 days (SD 1.147)**	**14.5 days (SD 1.100)**	**no**
P	**< 0.001**	**< 0.001**	**n.s.**

C GROUP: 20 patients who underwent to diode laser surgery without suture and used AMINOGAM® and 20 controls (not users) with Standard Deviation (SD)

	Lesion	clinical diameter	healing (median value)	complications
20 study cases	**fibro-epithelial**	**20 mm**	**17.5 days (SD 1.100)**	**no**
20 control cases	**fibro-epithelial**	**20 mm**	**21.1 days (SD 1.700)**	**15 % (candidosis)**

Data are expressed as median value. Comparison between control and case groups have been run by T-Test Student for impaired data or, when normality test failed, by Mann-Whitney Rank Sum Test. Comparison of proportion (for complications) has been evaluated by z-test, with Yates correction for small numbers. SigmaStat® software has been used for calculations.

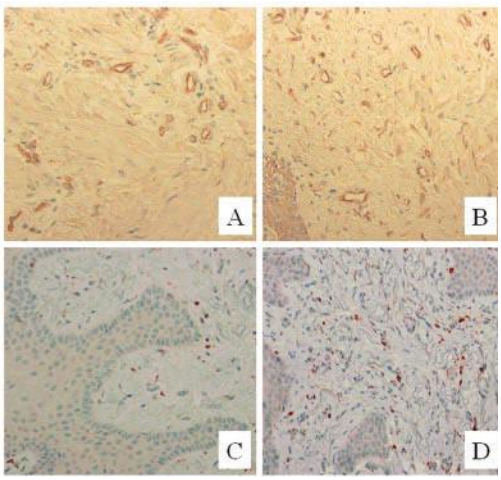

Fig. 1. *Immunohistochemical reactivity for CD31 (**A, B**) and CD68 (**C, D**) in bioptic specimens obtained from experimental (**B, D**) and control subgroup A (**A, C**). Note in the experimental subgroup a higher microvascular density and higher number of CD68-positive cells as compared to control one. Original magnification, 250 X.*

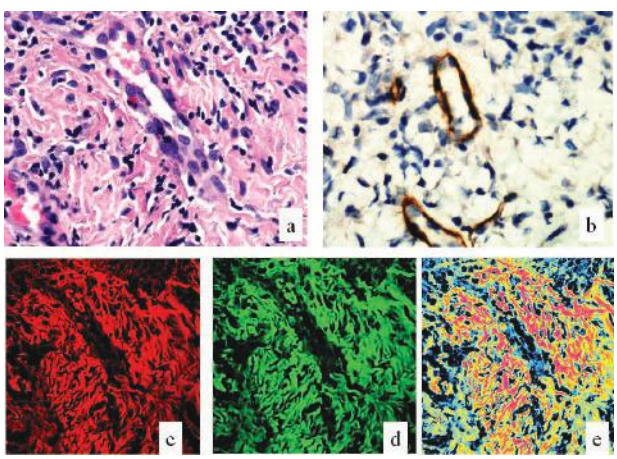

Fig. 2. *Collagen neo-synthesis in human gingiva treated with AMINOGAM®; tissue was obtained from patients of the experimental subgroup B (gingiva removed to expose endosseous implants after osteointegration); collagen deposition was observed above all around new formed vassels (**A**) as confirmed by immunohistochemical reactivity for collagen IV (**B**) and confocal laser scanning analysis (**C, D, E**).*

time between surgical procedure and suture removal (median time 6.9 days *vs* 7.6 days in the contralateral control sites) (p 0.007) was obtained. Moreover, in the experimental group faster healing (median time 11.5 days *vs* 14.4 days in the control group) ($p < 0.001$) of the extraction sockets with gingival keatinization was recognizable without post-surgical complications, whereas in 25% of the control subgroup delayed healing, post-extractive sicca alveolitis and infection were observed.

In the patients of subgroup B, who received endosseous dental implants with flap elevations and were treated with AMINOGAM® (three applications/die) until complete healing of the surgical incision, the removal of the suture was performed earlier (median time 6.9 days *vs* 10.5 days in control subgroup) ($p < 0.001$), gingival healing was faster (median time 8.4 days *vs* 14.5 days in the control subgroup) ($p < 0.001$), and no complications were found in either experimental or control subgroup.

In the patients of subgroup C, who received diode laser treatment of benign neoplasms of the oral mucosa without direct suture of the surgical margins and were treated with AMINOGAM® (three applications/die) until secondary healing, the healing time was shorter (median time 17.5 days *vs* 21.1 days in the control subgroup) ($p < 0.001$), without complications, whereas in 15% of the control group mycotic infections were observed.

In bioptic specimens obtained from patients of the experimental subgroup A, a significant increase of the microvascular density (mean value 12.5% *vs* 8.2%, $p < 0.001$ *vs* control) and of the number CD68-positive macrophage (mean value 12±4 *vs* 6±2, $p < 0.001$ *vs* control) as compared to the control subgroup was demonstrated (Fig. 1). In bioptic specimen obtained from patients of experimental subgroup B (gingiva removed to expose endosseous implants after osteointegration), a good collagen neo-synthesis, especially around new formed vassels, was observable in haematoxylin and eosin stains, confirmed by immunohistochemical findings on collagen IV stain and confocal laser scanning analysis (Fig. 2).

In vivo CAM assay

On day 12, macroscopic examination showed that sponges treated with AMINOGAM® were surrounded by numerous allantoic vessels, which developed radially towards the implant in a 'spoked-wheel' pattern (mean number of vessels around the implants 25±3) (Fig. 3). A similar picture was produced by sponges loaded with VEGF (mean

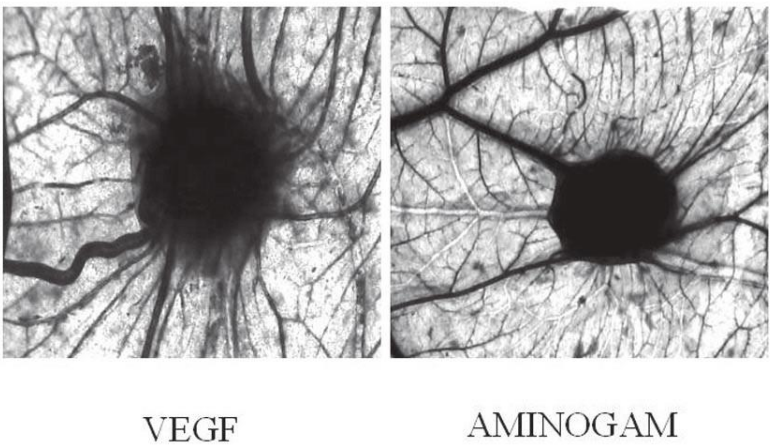

Fig. 3. *CAM at day 12 of incubation, 96 h after the AMINOGAM® treatment. Note the presence of numerous blood vessels converging toward the implant. The angiogenic response is similar to that induced by VEGF. Original magnification: x 50.*

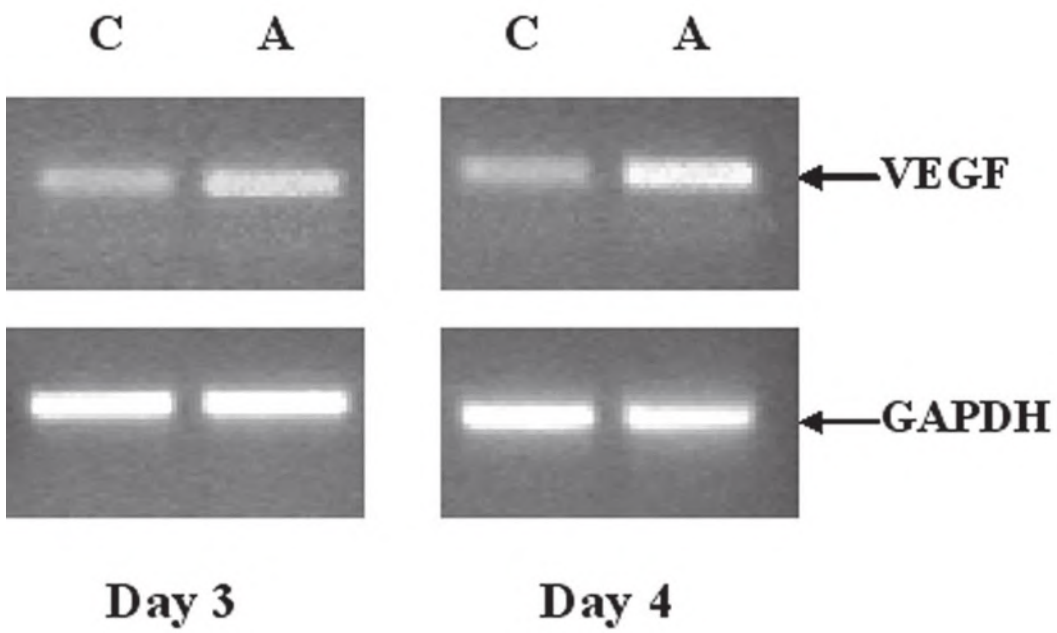

Fig. 4. *RT-PCR analysis of VEGF expression in MRC-5 at day 3 and 4 of incubation. C: untreated cells. A: cells treated with a solution of 0.0125 % aminoacids and 0.009 % sodium hyaluronate in H_2Od. VEGF expression raised in treated cells especially at day 4 of incubation.*

number of vessels around the implants 28±4) (Fig. 3). There was a statistically significant difference between AMINOGAM® and PBS loaded sponges (mean number of vessels around the implant 8±2, $p < 0.001$ *vs* AMINOGAM® treated sponges), whereas the angiogenic response induced by AMINOGAM® implants was comparable to that obtained with VEGF.

VEGF mRNA expression in human fibroblasts

The expression of VEGF gene in MRC-5 treated with AMINOGAM® solution was analyzed by RT-PCR. VEGF amplification product was slightly augmented on the third day, while a noteworthy rise was seen on the fourth day of incubation. All experiments were controlled by the presence of GAPDH expression (Fig. 4).

DISCUSSION

In this study for the first time we have demonstrated that a new medical substance, in form of a gel compound containing four aminoacids (glycine, leucine, proline, lysine) and sodium hyaluronate (AMINOGAM®), is able to accelerate the wound healing process of the soft oral tissues. To this purpose we studied three experimental conditions, dental extraction of molars on both side of the jaw, endosseous dental implants with flap elevations and diode laser treatment of benign neoplasms of the oral mucosa without direct suture of the surgical margins. In all the experimental conditions, AMINOGAM® treatment reduced the time of wound healing and was not associated to post-surgical complications, as compared to the same cases not treated with the compound. Moreover, in healing of gingival tissues covering endosseous implants after their insertion, an optimal gingival integrity obtained in a short time is essential to reduce infective complications which can lead to the damage and/or failure of implants. Moreover, it also promotes bone regeneration around the implants in the coronal portion, a critical zone for osteointegration and subsequently restoration. In the meantime, after diode laser surgery of soft oral tissues, the use of AMINOGAM® provides a double effect through mechanical protection of the wound and the acceleration of the healing process.

We also demonstrated by immunohistochemsitry a significant increase of the microvascular density and of the number CD68-positive macrophage in bioptic specimens obtained from patients of the first experimental subgroup as compared to those of the control subgroup, to confirm an increased vascularization and macrophage infiltration in the experimental specimens. Macrophages, in turn, may be involved in the angiogenic response taking place in the wound healing process, through the release of angiogenic cytokines, such as VEGF, contained in their cytoplasm.

To further investigate the possible direct angiogenic effect exerted by AMINOGAM®, we also tested this compound *in vivo* in the CAM assay and we demonstrated that AMINOGAM® stimulates angiogenesis, and that the angiogenic response is similar to that obtained with VEGF, a well-known angiogenic cytokine. Furthermore, AMINOGAM® solution is able to stimulate the expression of VEGF in human fibroblasts.

Among the components of AMINOGAM®, sodium hyaluronate is involved in angiogenesis. In fact, it enhances keratinocyte proliferation and migration, as well as the angiogenic response from the wound bed. In 1991, for the first time, West et al. (11) demonstrated that the degradation products of hyaluronic acid were pro-angiogenic. The angiogenic response was further confirmed (12-14) and then attributed to an intracellular effect upon signaling pathways (15-16) enhanced by co-application of VEGF (17). The involvement of hyaluronic acid in the regulation of angiogenesis is also supported by the observation that blocking the interaction of endogenous hyaluronic acid with cell surface hyaluronic acid-binding proteins inhibits both endothelial cell migration form the edge of a wounded monolayer and formation of tubular structures by endothelial cell clumps suspended in collagen gels (18).

Hyaluronic acid has been used extensively in a number of fields, including ophthalmic and joint surgery and, particularly, wound healing. Among the other components of AMINOGAM® there are four amino acids, namely glycine, leucine, proline and lysine, which seem to be involved in favoring wound healing by collagenogenesis and extracellular matrix formation (which is particularly promoted by the so-called Small Leucin Rich Proteoglicans

recently identified). In fact, the relationship between nutrition and wound healing, after injury or surgical intervention, has been recognized for centuries (19).

Finally, we have demonstrated that AMINOGAM® solution is able to stimulate the expression of VEGF in culture of human fibroblasts, also involved in the wound healing process. We hypothesize that fibroblasts and macrophages, which enter the composition of the cellular infiltrate in wound healing, may be involved in the stimulation of angiogenesis through the release of a pro-angiogenic cytokine, such as VEGF.

Overall, these clinical and experimental data, indicating that AMINOGAM® accelerates wound healing, promote angiogenesis and the release of VEGF by fibroblasts, suggest that this new compound may be successfully used in the treatment of several oral pathological conditions in which an accelerated repair of the surgical solution is of benefit for the patient.

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