European Review for Medical and Pharmacological Sciences 2013; 17: 2721-2727

Multicenter clinical trial on the performance and tolerability of the Hyaluronic acid-collagenase ointment for the treatment of chronic venous ulcers: a preliminary pilot study

G. GRAVANTE[1], R. SORGE[2], N. GIORDAN[3], S.R. GEORGESCU[4], S.H. MORARIU[5], I. STOICESCU[6], V. CLATICI

[1]Department of Surgery, Northampton General Hospital, Northampton, United Kingdom
[2]Department of Human Physiology, Laboratory of Biometry, University of Tor Vergata, Rome, Italy
[3]Fidia Farmaceutici S.p.A., Abano Terme, Italy
[4]Spitalul Clinic Dermato, Venerice "Prof. Dott. Scarlat Ionghin", Bucuresti, Romania
[5]Spitalul Clinic Municipal, Targu Mures, Romania
[6]Clinica de Dermatologie, Spitalul Clinic Judetean Craiova, Craiova, Romania
[7]Spitalul Universitar Elias, Bucuresti, Romania

Abstract. – BACKGROUND: Bed debridement is important to treat chronic wounds. Effective agents should remove the necrosis but protect the granulation tissue. We evaluated the performance and tolerability of a new composite ointment containing collagenase and hyaluronic acid for chronic venous ulcers.

PATIENTS AND METHODS: Subjects with class 6 venous ulcers (CEAP classification) of at least 6 months duration were prospectively recruited. The ointment was administered daily and follow-up visits were conducted on the fifth, 10th, 15th and 20th days. On each visit the necrotic area was measured with a grid. The moisture balance, odour, viability of non-necrotic areas and the presence of erythema were also assessed. Primary outcome was the percentage of subjects with complete debridement, secondary outcomes the time to complete healing, reduction of the lesion area, absence of necrotic tissue, presence of odor, erythema, hydration, any adverse events.

RESULTS: One hundred subjects were enrolled in four centres. All patients achieved complete debridement of the necrotic area and a significant reduction of the total ulcer area by day 20, while other parameters improved significantly over time. Only two patients experienced a transient leg oedema.

CONCLUSIONS: The combination of collagenase and hyaluronic acid is safe and effective for chronic venous ulcers.

Key Words:
Venous ulcer, Hyaluronic acid, Collagenase.

Introduction

Venous ulcers affect 0.3-2% of the general population and 2-4% of patients with chronic venous insufficiency[1-4]. Among all ulcerations up to one-third become chronic[5] and may recur after treatment[2]. Factors associated with poor healing and recurrence are both patient-related (male sex, advanced age, increased BMI, diabetes, history of DVT (deep venous thrombosis), non-compliance with compression therapy)[6,7] and local (i.e., the ulcer dimensions, oxygenation status, duration of the ulcer)[6]. Peculiar histological and biochemical modifications of the local milieu also contribute to the ulcer tendency to persist. Alterations of the cellular functions activate the production of cytokines and matrix metalloproteinases and lead to a status of non-resolving chronic inflammation[8-10]. More interestingly, such modifications morphologically organise with a "target" appearance around the chronic ulcer[11]: the first inner zone adjacent to the ulcer corresponds to an area of acute purulent inflammation, the middle zone to granulative-proliferative inflammation with organisation of the purulent exudates and the outer zone to fibrous connective tissue with inflammatory cellular infiltration in it[11].

Although the mainstay of treatment for chronic venous ulcers is still based on compressive bandages[12], it is still important to prepare the bed wound properly to furnish the ulcer with the highest possibility to heal. However, the simulta-

Corresponding Author: Gianpiero Gravante, Ph.D.; e-mail: ggravante@hotmail.com

neous presence in the ulcer bed of negative factors (the fibrinous tissue) and positive factors for healing (granulation) renders the local treatment more difficult. In fact, it is imperative to remove the fibrin and dead cells but also at the same time to stimulate the production of granulation tissue. These tasks are opposite and cannot be achieved easily by dressings containing only one product. Topical preparations that combine two or more products already exist in clinical practice (i.e., alginate with an antimicrobial and hydrogel, silver with alginate, collagen with a hydrocolloid, collagenase with an antimicrobial)[13,14] but are not specific for this purpose. Therefore, we evaluated the safety and efficacy of the combination of collagenase (for the enzymatic debridement of necrotic and fibrinous tissue) and hyaluronic acid (HA – for stimulation of healing) in the same ointment to treat chronic venous ulcers.

Patients and Methods

The study started after the approval released by the Romanian Health Ministry (nr. 12 din 28.12.2009, amended and approved 23938/09.04.10 and 30127/19.05.10) and by the local Ethics Committees of the four centres participating in the trial (Spitalul Clinic Dermato, Venerice "Prof. Dott. Scarlat Ionghin", Bucuresti – Romania; Spitalul Clinic Municipal, Targu Mures, Romania; Clinica de Dermatologie, Spitalul Clinic Judetean Craiova, Craiova, Romania; Spitalul Universitar Elias, Bucuresti, Romania – International Clinical Trial Registration: ISRCTN12579869). The investigator explained the nature, purpose and risks of the study and provided the patient with a copy of the information sheets in Romanian language approved by the local Ethics Committee. The written informed consent was, therefore, obtained before the patient entered the study.

Inclusion criteria consisted of subjects between 18 and 80 years of age with class 6 chronic venous ulcers (CEAP classification), of at least 6 months of duration, total lesion area between 2 and 45 cm^2 and necrosis area greater than 35% of the total. Excluded from the study were venous leg ulcers with dry black eschars, exposed bones, tendons or fascia, subjects with Ankle Brachial Pressure Index (ABPI) lower than 0.8 (measured by Doppler sonography) or peripheral arterial disease, subjects using occlusive wound dressings (elastocompressive bandages were allowed), local antibiotics, hydrogels, hydrocolloids, detergents, with known hypersensitivity to collagenase or HA, immunocompromised subjects or affected by severe systemic conditions, severe obesity (Body Mass Index > 35). The enrolment was conducted at four clinical sites in Romania.

Experimental Protocol

Bionect Start® (Fidia Farmaceutici S.p.A., Abano Terme, Italy) is a topical ointment based on hyaluronic acid sodium salt by fermentation (0.2% w/w), and bacterial collagenase obtained from non-pathogenic *Vibrio alginolitycus* (> 2.0 nkat/g). A layer of about 2 mm thickness was applied on the wound bed once a day. Prior to application the wound was cleansed of debris by gently rubbing with a gauze pad saturated with normal saline solution. Dry wounds had to be moistened with physiological saline (0.9% NaCl) prior to treatment. The treatment was covered using a non-occlusive dressing to assure the contact with the wound surface, followed by application of a firm compression bandage or a graduated elastic medical compression stocking to the leg with the venous ulcer. Treatment continued until complete wound debridement or for a maximum period of 20 days if complete wound debridement was not reached before. Wounds were diagnosed as completely healed when the normal re-epithelization process was complete and covered all affected areas. Follow-up visits were conducted on the 5^{th}, 10^{th}, 15^{th} and 20^{th} day after the start of the treatment. During each visit the necrotic area was measured with a grid while odour, wound bed moisture, viability of non-necrotic areas and the presence of erythema or redness were assessed clinically. Laboratory determinations for haematology and blood chemistry were also recorded at baseline visit and at the final visit.

Outcomes

Primary outcome of the study was the number of subjects with complete debridement at the end of the treatment. The debridement was evaluated creating a 5-points scale that divided into classes the percentage of necrotic area. The percentage of necrotic area was calculated dividing the necrotic area expressed in cm^2 by the total ulcer area expressed in cm^2 and the result reported as a percentage[15]. The 5-points scale was: 1 = 0% necrotic area, 2 = 1%-25% necrotic area, 3 = 26%-50%, 4 = 51%-75%, 5 = 76%-100%. Patients with successful debridement were considered those of class 1 or 2 (0-25% necrotic area out of the total area).

Secondary outcomes were the reduction of the total ulcer area, necrotic area, percentage of necrotic area, odour (absent, minimal, tolerable, intense, repulsive), moisture balance (very poor; poor; satisfactory; good; very good), tissue viability (very poor; poor; satisfactory; good; very good), presence of erythema or redness (absent, minimal, moderate, abundant)[15]. Furthermore, the easiness of application, pain during the change of dressing, and any undesired systemic or local effects were also evaluated and recorded. The pain was initially scored with a Visual Analogic Scale (VAS, 0-10) and then reported in classes as absent (VAS = 0), minimal (VAS = 1-3), bearable (VAS = 4-6), intense (VAS7-9), unbearable (VAS = 10).

Sample Size Estimation

The sample size was calculated using the NCSS 2007 Statistical & Power Analysis Software (Kaysville, UT, USA). For the calculation we adopted the reduction of necrotic area of the ulcer as a gold-standard parameter. Considering the initial necrotic area as 100% value, a reduction of 70% at the end of the treatment, a non-Gaussian distribution of the area and an alpha value of 0.05, we obtained a power of 99% with a sample size of 55 subjects. As patients were followed in Clinics it was anticipated a drop-out rate of 30%. Therefore, the minimum number of patients recruited in the study was 55+17 = 72 patients.

Statistical Analysis

The database was constructed with Excel (Microsoft Corporation, Redmond, WA, USA) and the statistical analysis performed using the Statistical Package for the Social Sciences Windows version 15.0 (SPSS Inc., Chicago, IL, USA). Descriptive statistics for quantitative variables were the mean and standard deviation (mean ± SD) for parametric variables or median and range (minimum and maximum) for non-parametric variables. Normality assumptions were demonstrated with the Kolmogorov/Smirnov testing. Descriptive statistics for qualitative variables was performed with occurrences and described with relative frequencies.

The evolutions of the ulcer areas over time have been analysed using the Friedman test. Ordinal variables were compared with the Chi-square test (Fisher's exact test if occurrences in cells were inferior to 5). All results were considered significant if $p < 0.05$.

Results

Between February and November 2010 one hundred patients were recruited and followed-up for the study. Fifty-three were males (53%), mean age was 66 ± 10 years, height 170 ± 10 cm, weight 80 ± 15 kg, Body Mass Index 28 ± 4 kg/m^2, ABPI 0.84 (range 0.80-1.00). The ulcer was already present for 240 days (range 180-730) before the start of the treatment.

Patients with a successful debridement (class 1 and 2) significantly increased during the treatment (Fisher's exact test, $p < 0.001$) and constituted two thirds of our series by the 10th day of applications (Table I). The descriptive statistics for ulcer areas are presented in Table I, their graphic evolution over time in Figures 1 and 2.

Table I. Descriptive statistics of ulcer areas over time and results obtained after classifying patients according to the 5-points scale of debridement.

		Time of visit (days)				
		0	5	10	15	20
Scale of debridement	1	0	4 (4%)	15 (15%)	29 (29%)	100 (100%)
	2	0	26 (26%)	50 (50%)	66 (66%)	0
	3	11 (11%)	51 (51%)	35 (35%)	5 (5%)	0
	4	47 (47%)	17 (17%)	0	0	0
	5	42 (42%)	2 (2%)	0	0	0
Total area (cm^2)		11 (3-41)	9 (0-38)	8 (0-32)	7 (0-32)	6 (0-32)
Necrotic area (cm^2)		7 (2-27)	3 (0-17)	2 (0-8)	1 (0-4)	0
Percentage of necrotic area (%)		70 (35-96)	31 (0-81)	19 (0-49)	9 (0-39)	0

The scale of debridement represents the percentage of necrotic area classified in a five-point scale. 1 = 0% necrotic area, 2 = < 25% of necrotic area, 3 = 26%-50%, 4 = 51%-74%, 5 = 75%-100%.

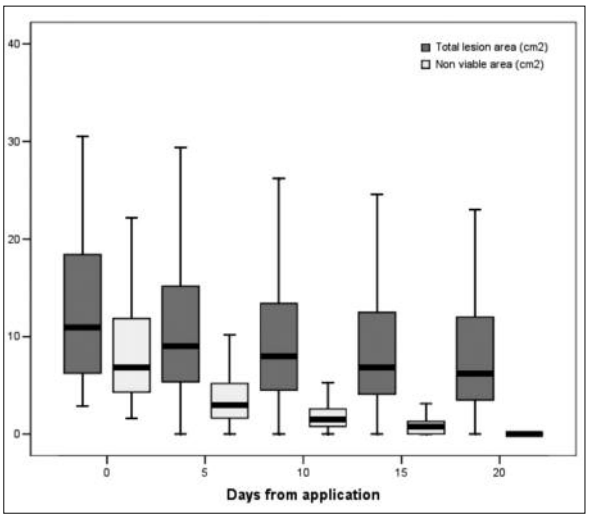

Figure 1. Box plots representing the evolution of total and necrotic areas during the treatment. Boxes represent the 95% Confidence Intervals, the black line the median value, whiskers the minimum and maximum values.

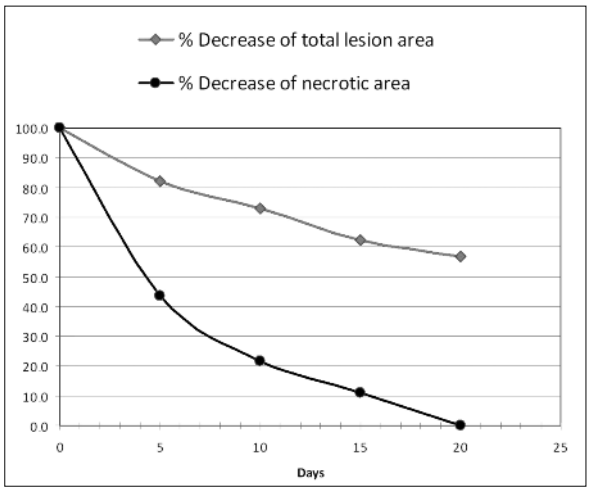

Figure 2. Trends of the percentage of necrotic area (black line) and total ulcer area (red line) during the treatment. Boxes represent the 95% Confidence Intervals, the black line the median value, whiskers the minimum and maximum values.

The total ulcer area significantly decreased from the 10th day of application onwards compared to baseline pre-treatment levels ($p = 0.004$), the necrotic area and the necrotic percentage from the 5th day (Friedman test, $p < 0.001$). The necrotic component of the ulcer decreased at a higher rate compared to the total area (Figure 2).

A significant improvement was observed over time for qualitative parameters of the ulcer such as odour, erythema, tissue viability and moisture balance (Table II). A significant progressive shift towards better classes was found during the treatment compared to baseline levels: odour (Fisher's exact test, $p < 0.001$), erythema (Fisher's exact test, $p < 0.001$), tissue viability (Fisher's exact test, $p < 0.001$), moisture balance (Fisher's exact test, $p < 0.001$) (Table II).

Table II. Description of the ulcer qualitative parameters evaluated (see text for an explanation of the classes adopted).

		Time of visit (days)				
	Class	0	5	10	15	20
Odour	1	27 (27%)	52 (52%)	78 (78%)	100 (100%)	100
	2	20 (20%)	28 (28%)	20 (20%)	0	0
	3	29 (29%)	20 (20%)	2 (2%)	0	0
	4	24 (24%)	0	0	0	0
Erythema	1	21 (21%)	31 (31%)	44 (44%)	61 (61%)	95 (95%)
	2	10 (10%)	50 (50%)	52 (52%)	39 (39%)	5 (5%)
	3	38 (38%)	18 (18%)	4 (4%)	0	0
	4	31 (31%)	1 (1%)	0	0	0
Tissue viability	1	1 (1%)	0	0	0	0
	2	12 (12%)	3 (3%)	0	0	0
	3	62 (62%)	71 (71%)	57 (57%)	39 (39%)	32 (32%)
	4	24 (24%)	24 (24%)	33 (33%)	57 (57%)	54 (54%)
	5	1 (1%)	2 (2%)	11 (11%)	4 (4%)	14 (14%)
Moisture balance	1	1 (1%)	0	0	0	0
	2	12 (12%)	4 (4%)	0	0	0
	3	58 (58%)	68 (68%)	48 (48%)	31 (31%)	31 (31%)
	4	28 (28%)	27 (27%)	46 (46%)	65 (65%)	56 (56%)
	5	1 (1%)	1 (1%)	6 (6%)	4 (4%)	13 (13%)

All researchers involved in the application of the dressing found that this was easy to apply throughout the study. Thirteen patients (13%) complained of minimal pain during the dressing removal and new application (VAS = 1-3) while 87% of them referred no pain (VAS = 0). Considering adverse effects, only two patients (2%) experienced a monolateral mild oedema of the leg for 5 and 12 days during the treatment. Oedemas spontaneously resolved without discontinuation of the local application.

Discussion

Wound bed preparation continues to be an essential component of chronic wound management. By addressing the relationship between necrotic tissues, exudates, bacteria and cellular dysfunction an optimal wound healing environment can be achieved[16]. Enzymatic debridement is only one of the available methods to remove necrotic tissues but is frequently used in the long-term care due to immediate availability, easiness to administer and safety[17]. Furthermore, it also harbours the theoretical advantage of furnish to the local tissues small peptides of collagen from the digestion of necrotic materials that act as chemoattractive substances promoting cell proliferation and movement. Collagenase is the best characterized of all of the enzymatic debriding agents. It specifically digests all triple helical collagen and does not degrade other proteins lacking of the triple helix conformation[18]. This is a unique feature of bacterial collagenase since none of the other available proteases can digest collagen[19,20]. It has been used for over 25 years and presented a large number of clinical advantages including the selective removal of dead tissue, is a painless ointment, and causes minimal amount of blood loss. This type of debridement is appropriate for the use in long-term care facilities or at home[19,20].

Although debridement is an important part of wound care, information to guide evidence-based decisions is limited in the literature where studies are based on small series and involve heterogeneous patients with predictable bias[21]. According to this trials, collagenase achieved an average healing time of 18-24 days on partial thickness burns[13,22], longer in chronic ulcers (9-15 weeks)[22]. Considering pressure sores, complete wound debridement was obtained mainly by day 42 of treatment[23]. Therefore, we originated the hypothesis to combine the enzymatic debridement with a product able to stimulate the tissue regeneration in order to improve the literature results. Our previous experience with the HA suggested that this product could be the ideal candidate to be combined with the collagenase[24,25].

HA was first isolated in 1934 from the bovine vitreous humour but was subsequently found also in soft connective tissues, synovial fluids, umbilical cords and rooster combs. It is one of the main extra cellular matrix (ECM) components and consists of a non-sulphated, linear glycosaminoglycan with repeated units of glucuronic acid and N-acetyl-glucosamine[26-30]. The physiological functions derive from its structural role in the ECM and from the ability to interact with cell surface receptors. HA is a hydrophilic molecule which binds a large amount of water and forms a viscous hydrated gels even at low concentrations[31]. While the peculiar hygroscopic and rheological properties allow for a good hydration of the ECM and tissue elasticity, the cell-cell and cell-substrate adhesions consent the HA to interact with the cellular microenvironment leading to cellular proliferations, migrations and ECM deposition. Because of its porous and hydrated organisation, HA allows the rapid diffusion of water-soluble molecules and migration of cells during the wound repair process[31]. For all these reasons, the main role of HA in tissue repair processes consists in facilitating the entry of a large number of cells into the injured area and in orientating the deposition of ECM fibrous components[26-30]. Furthermore, HA seems to protect directly the granulation tissue from oxygen-free radicals that impair the wound healing, possibly acting as a molecular scavenger[32,33].

Although sporadic reports have already described the positive effects of HA on chronic venous ulcers, these studies had a low sample size (n = 10)[34] or compared HA vs. dextranomer[35]. Only recently two multicentric randomised controlled trials (RCTs) compared HA vs. placebo for the treatment of chronic venous leg ulcers[36,37]. HA achieved a 27%-40% reduction of the total area of the ulcer (viable + non-viable component) at the 15th day of treatment compared to placebo where the reduction was lower (6%-29%)[36,37]. In our study Bionect Start® had a similar effect on the total area of the ulcer where the reduction corresponded at the 15th day of treatment to 36% of the initial surface (4/11 cm^2). However, the greatest difference between Bionect Start® and published re-

sults of HA alone involves the non-viable portion of the ulcer. While HA alone showed a decrease from 41% at day 1 of treatment to 31% at day 15[36], Bionect Start® decreased the non-viable area from an initial 70% at day 0 to 9% at day 15. It is possible that the greater decrease of the non-viable area observed in our study derives from the presence of collagenase, not present in the two RCTs[36,37], This could have increased the local beneficial effects of HA on wound healing by digesting the necrotic part.

Few limitations need to be acknowledged by the reader. First, the study is an observational longitudinal without a comparison arm to evaluate the real effects of the combination of the two products (collagenase and HA) over each alone. As this was a preliminary pilot study for the evaluation of safety and potential efficacy of the Bionect Start®, it needs now to be followed by further randomised trials possibly comparing HA + collagenase vs. HA vs. collagenase vs. placebo (four-arm trial). Such studies should also include elastocompressive bandages in all their arms otherwise these could bias the results of one product over the others. A second limitation involved the lack of any histological and especially immunohistochemical evaluation of the regenerative activity of HA + collagenase. Again, this could be a point for future researches especially compared to HA or collagenase alone. Finally, the lack of a long-term follow-up does not allow us to present any data on long-term recurrences.

Conclusions

Bionect Start®, a new ointment formulation of collagenase with HA, was shown to be an effective debriding agent for the treatment of chronic venous ulcers. During the study all patients reached a complete debridement with a significant reduction of the total and necrotic lesion areas. Furthermore, all the other parameters associated with the ulcer (odour, erythema, tissue viability and moisture balance) showed a significant improvement over time. The safety of the device appeared globally satisfactory and no sensitivity reactions were reported.

Conflict of Interest

The Authors declare that they have no conflict of interests.

References

1) Maffei FH, Magaldi C, Pinho SZ, Lastoria S, Pinho W, Yoshida WB, Rollo HA. Varicose veins and chronic venous insufficiency in Brazil: prevalence among 1755 inhabitants of a country town. Int J Epidemiol 1986; 15: 210-217.
2) Nelzen O, Bergqvist D, Lindhagen A. Venous and non-venous leg ulcers: clinical history and appearance in a population study. Br J Surg 1994; 81: 182-187.
3) Valencia IC, Falabella A, Kirsner RS, Eaglstein WH. Chronic venous insufficiency and venous leg ulceration. J Am Acad Dermatol 2001; 44: 401-421; quiz 422-404.
4) Reichenberg J, Davis M. Venous ulcers. Semin Cutan Med Surg 2005; 24: 216-226.
5) Callam MJ, Ruckley CV, Harper DR, Dale JJ. Chronic ulceration of the leg: extent of the problem and provision of care. Br Med J (Clin Res Ed) 1985; 290: 1855-1856.
6) Labropoulos N, Wang ED, Lanier ST, Khan SU. Factors Associated with poor healing and recurrence of venous ulceration. Plast Reconstr Surg 2012; 129: 179-186.
7) van Ruswijk L. Full-thickness leg ulcers: patient demographics and predictors of healing. Multi-Center Leg Ulcer Study Group. J Fam Pract 1993; 36: 625-632.
8) Raffetto JD, Marston WA. Venous ulcer: what is new? Plast Reconstr Surg 2011; 127(Suppl 1): 279S-288S.
9) Moor AN, Vachon DJ, Gould LJ. Proteolytic activity in wound fluids and tissues derived from chronic venous leg ulcers. Wound Repair Regen 2009; 17: 832-839.
10) Herouy Y, Trefzer D, Zimpfer U, Schopf E, Wanscheidt W, Norgauer J. Matrix metalloproteinases and venous leg ulceration. Eur J Dermatol 2000; 10: 173-180.
11) Arabidze G, Chkhaidze H. Pathomorphological peculiarities of trophic ulcer developed during chronic venous insufficiency of the lower limbs. Georgian Med News 2008; 163: 58-61.
12) Palfreyman S, King B, Walsh B. A review of the treatment for venous leg ulcers. Br J Nurs 2007; 16: S6-14.
13) Hansbrough JF, Achauer B, Dawson J, Himel H, Luterman A, Slater H, Levenson S, Salzberg CA, Hansbrough WB, Dore C. Wound healing in partial-thickness burn wounds treated with collagenase ointment versus silver sulfadiazine cream. J Burn Care Rehabil 1995; 16: 241-247.
14) Soroff HS, Sasvary DH. Collagenase ointment and polymyxin B sulfate/bacitracin spray versus silver sulfadiazine cream in partial-thickness burns: a pilot study. J Burn Care Rehabil 1994; 15: 13-17.
15) Alvarez OM, Fernandez-Obregon A, Rogers RS, Bergamo L, Masso J, Black M. A prospective, randomised, comparative study of collagenase and papaine-urea for pressure ulcer debridement. Wounds 2002; 14: 293-301.

16) Lazarus GS, Cooper DM, Knighton DR, Margolis DJ, Pecoraro RE, Rodeheaver G, Robson MC. Definitions and guidelines for assessment of wounds and evaluation of healing. Arch Dermatol 1994; 130: 489-493.

17) Ramundo J, Gray M. Enzymatic wound debridement. J Wound Ostomy Continence Nurs 2008; 35: 273-280.

18) Silverstein P, Ruzicka FJ, Helmkamp GM, Jr., Lincoln RA, Jr., Mason AD, Jr. *In vitro* evaluation of enzymatic debridement of burn wound eschar. Surgery 1973; 73: 15-22.

19) Enoch S, Harding KG. Wound bed preparation – the science behind the removal of barriers to healing. Wounds 2003; 15: 213-229.

20) Altman MI, Goldstein L, Horowitz S. Collagenase: an adjunct to healing trophic ulcerations in the diabetic patient. J Am Podiatry Assoc 1978; 68: 11-15.

21) Smith RG. Enzymatic debriding agents: an evaluation of the medical literature. Ostomy Wound Manage 2008; 54: 16-34.

22) Marazzi M, Stefani A, Chiaratti A, Ordanini MN, Falcone L, Rapisarda V. Effect of enzymatic debridement with collagenase on acute and chronic hard-to-heal wounds. J Wound Care 2006; 15: 222-227.

23) Milne C, Ciccarelli A, Lassy M. A Comparison of collagenase to hydrogel dressings in wound debridement. Wounds 2010; 22: 270-274.

24) Gravante G, Delogu D, Giordan N, Morano G, Montone A, Esposito G. The use of Hyalomatrix PA in the treatment of deep partial-thickness burns. J Burn Care Res 2007; 28: 269-274.

25) Gravante G, Sorge R, Merone A, Tamisani AM, Di Lonardo A, Scalise A, Doneddu G, Melandri D, Stracuzzi G, Onesti MG, Cerulli P, Pinn R, Esposito G. Hyalomatrix PA in burn care practice: results from a national retrospective survey, 2005 to 2006. Ann Plast Surg 2010; 64: 69-79.

26) Manuskiatti W, Maibach HI. Hyaluronic acid and skin: wound healing and aging. Int J Dermatol 1996; 35: 539-544.

27) King SR, Hickerson WL, Proctor KG. Beneficial actions of exogenous hyaluronic acid on wound healing. Surgery 1991; 109: 76-84.

28) Davidson JM, Nanney LB, Broadley KN, Whitsett JS, Aquino AM, Beccaro M, Rastrelli A. Hyaluronate derivatives and their application to wound healing: preliminary observations. Clin Mater 1991; 8: 171-177.

29) Campoccia D, Hunt JA, Doherty PJ, Zhong SP, O'Regan M, Benedetti L, Williams DF. Quantitative assessment of the tissue response to films of hyaluronan derivatives. Biomaterials 1996; 17: 963-975.

30) Costagliola M, Agrosi M. Second-degree burns: a comparative, multicenter, randomized trial of hyaluronic acid plus silver sulfadiazine vs. silver sulfadiazine alone. Curr Med Res Opin 2005; 21: 1235-1240.

31) Lindahl U, Hook M. Glycosaminoglycans and their binding to biological macromolecules. Annu Rev Biochem 1978; 47: 385-417.

32) Foschi D, Castoldi L, Radaelli E, Abelli P, Calderini G, Rastrelli A, Mariscotti C, Marazzi M, Trabucchi E. Hyaluronic acid prevents oxygen free-radical damage to granulation tissue: a study in rats. Int J Tissue React 1990; 12: 333-339.

33) Trabucchi E, Pallotta S, Morini M, Corsi F, Franceschini R, Casiraghi A, Pravettoni A, Foschi D, Minghetti P. Low molecular weight hyaluronic acid prevents oxygen free radical damage to granulation tissue during wound healing. Int J Tissue React 2002; 24: 65-71.

34) Mekkes JR, Nahuys M. Induction of granulation tissue formation in chronic wounds by hyaluronic acid. Wounds 2001; 13: 159-164.

35) Ortonne JP. Comparative study of the activity of hyaluronic acid and dextranomer in the treatment of leg ulcers of venous origin. Ann Dermatol Venereol 2001; Suppl: 13-16.

36) Dereure O, Czubek M, Combemale P. Efficacy and safety of hyaluronic acid in treatment of leg ulcers: a double-blind RCT. J Wound Care 2012; 21: 131-132, 134-136, 138-139.

37) Humbert P, Mikosinki J, Benchikhi H, Allaert FA. Efficacy and safety of a gauze pad containing hyaluronic acid in treatment of leg ulcers of venous or mixed origin: a double-blind, randomised, controlled trial. Int Wound J 2012; 10: 159-166.