A NEW PHARMACEUTICAL DEVICE CONTAINING MESOGLYCAN MODULATES FIBROBLASTS FUNCTION IN VIVO

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Abstract

Our recent investigations showed in vitro that a new pharmaceutical device containing mesoglycan, the Prisma® Skin, enhances re-epithelialization and granulation processes. This device induces a strong cytoskeletal re-organization to enhance cell migration/invasion, showing the increase of Fibroblast Activated Protein α and a remarkable change in fibroblast shape and orientation.

In this case series we obtained preliminary evidences about the beneficial effects of Prisma® Skin device on patients. This pilot study was performed on seven permanently bedded patients affected by pressure ulcers and focusing on fibroblast activity, to confirm our in vitro results. The haematoxylin-eosin and immunohistochemical staining of patients’ biopsies have been used to assess the relevant cell structures of tissue samples.

We started the evaluation at a T0 point proved the lack of cell structure in the analyzed tissues with a tendency to wound bed sclerosis, and reduced inflammatory activity. Then, after 14 days of treatment with Prisma® Skin, we founded a relevant formation of granulation tissue. The strong increase of vimentin expression confirmed not the wound closure than a strong fibroblasts recruitment on patients’ skin biopsies.

Our clinical preliminary study shows, for the first time, the positive effects on tissue repair of Prisma® Skin on patients’ pressure ulcers.

Keywords: mesoglycan, glycosaminoglycans, skin wound healing, pressure ulcers, wound care.
Introduction

It has been shown that the use of dressings containing hyaluronic acid (HA) and other compounds of the extracellular matrix (ECM) may prove beneficial in non-healing skin ulcers [1, 2]. These dressings are able to release the ECM proteins and growth factors in the wound bed [3]. These devices could function as bio-stimulators and as they can be considered as natural contributors to the healing process, it is conceivable that they could be used in a practical and safe manner. In this scenario, it can be included the Prisma® Skin, the pharmaceutical dressing device developed by Mediolanum Farmaceutici s.p.a., thanks to the presence of mesoglycan [4]. This latter is a natural glycosaminoglycans (GAG) preparation extracted from porcine intestinal mucosa and is composed of heparan-sulphate (HS) (47.5%), dermatan-sulphate (DS) (35.5%), slow-moving heparin (HEP) (8.5%), and chondroitin-sulphate (CS) (8.5%). Several clinical studies reported the efficacy of mesoglycan in the treatment of vascular diseases associated or not to thrombotic risks such as deep venous thrombosis and chronic venous insufficiency [5-8].

GAGs represent a very heterogeneous and important family of macromolecules deriving from carbohydrates as uronic acids or L-iduronic acid and amino-sugars such as glucosamine and galactosamine. The most important components of this family are HA, CS, DS, keratan-sulphate (KS), HS and HEP [9]. Except HA, GAGs constitute the lateral chains of protein structure to form the Proteoglycans (PGs) [10]. During wound repair, GAGs play many and varied roles in all stages, providing architectural support and hydration to tissues favourable to cellular migration into the wound site to promote repair processes. Notably, specific molecular mechanisms have been identified for GAGs and PGs in each phase of wound repair, including granulation, re-epithelialization and angiogenesis [11].

Furthermore, our recent in vitro investigations showed that Prisma® Skin is able to enhance re-epithelialization and granulation processes, acting on human epidermal keratinocytes and dermal fibroblasts. Interestingly, it has been shown that mesoglycan can induce a strong cytoskeletal re-organization to increase cell migration and invasion, two key processes at the base of the re-epithelialization and granulation of wound healing [12-16]. We also showed by immunofluorescence assay, that fibroblast treated with mesoglycan exhibited the increase of Fibroblast Activated Protein (FAP)-α and a remarkable change in shape and orientation, two common features of reactive stromal fibroblasts.

Mesoglycan and the whole Prisma® Skin are also able to positively affect the angiogenesis enhancing the in vitro formation of new blood vessels. The identified mechanism includes the induction of endothelial-to-mesenchymal transition by which endothelial cells acquire a fibroblast-like phenotype and become able to migrate, invade and form new capillary-like structures. Finally, Prisma® Skin is able to regulate inflammatory responses which are required in the first stages of wound repair but can later induce damage because of dermal, epidermal and endothelial cells recruitment [17].

In order to confirm the results previously obtained in vitro, we had to define the clinical and histological condition in a skin ulcer suitable for such evaluation. Unlike other types of skin ulcers, pressure ulcers are determined mainly by immobility, independently from the patients’ clinical features and somatic characteristics [18]. Pressure ulcers evolve through several degrees of severity, which can be accurately evaluated considering the topography, the staging and the condition [19], according to international classifications.

Taken together, these features have made pressure ulcers as a good clinical model to evaluate in vivo the effects of Prisma® Skin. Particularly, the proliferative arrest has been the starting point to study the fibroblasts activity highlighting the function of this device on the granulation phase of wound repair.

Methods

Patients recruitment

Seven patients have been selected as affected by pressure ulcers in home wound care from almost three months. The lesions appeared cleansed, without any signs of bacterial infection, in phase of proliferative arrest from at least three weeks. This aspect becomes clinically evident, (no reduction of lesion area). All the pressure ulcers selected had to be overlapping for region (sacral), stage (III) and
status (cleansed and/or tending towards wound bed sclerosis).

The criteria for the exclusion were:

- neoplastic diseases;
- enteral and parenteral nutrition;
- albumin <3.5 g/dl;
- lymphocytes <1500;
- infected lesion;
- other types of skin ulcers beside the pressure ones;
- not accurately performed mobilization of patient by nurses.

The patients inserted in this study have suspended the previous medical treatment and have been medicated using the following method:

- cleansing with saline solution and povidone-iodine (50:50);
- Prisma® Skin as primary dressing;
- sterile gauze as secondary dressing;
- time of dressing change: 24h.

Skin biopsy collection

The area for the biopsies is identified in a not undermined region between 0.5 and 1 cm far from the lesion’s edge. The biopsies have been performed at T0 and T14 (after 14 days from the beginning of the treatment). At T14 the tissues have been collected in a distant area from the previous point of biopsies at T0, on a parallel board at the lesion’s edge between 1 and 2 cm. This procedure was used to avoid activity of tissue repair process after biopsy in that area. The biopsies have been performed with a punch of a diameter of 3.5 mm. At T0 and T14 a photo feature has been carried out to compare the histological data with the clinical ones. All the harvested samples have been analyzed in double-blinded, without any clinical information about patients and experimental time.

H&E tissue staining

The harvested tissues were differently treated for H&E and IHC staining. In the first case, the samples were treated as reported in [20]. Briefly, they were fixed in a solution of formaldehyde 0.5% in PSB (Phosphate-Buffered Saline) 1x for 2 hours at 4°C. Then they were incubated O/N at 4°C in a sucrose solution (15% w/v in PBS 1x) to guarantee the cryoprotection. Next, the samples were included in OCT (Optimal Cutting Temperature; Sakura Finetek, Flemingweg, Netherlands) and stored at -80°C. The frozen tissue sections were cut on a microtome RM2125RT (Leica Microsystems, Wetzlar, Germany), mounted directly on super frost slides (Thermo Scientific, Waltham, MA), and processed for haematoxylin and eosin (H&E) staining. Briefly, cryostat sections were dehydrated for 5 minutes with cold acetone and then rehydrated. Next, slides were placed in haematoxylin stain for 9 minutes, rinsed in alcoholic acid, differentiated in 80% alcohol and stained with eosin for 2.5 minutes, rinsed in 95% ethanol, dehydrated with absolute ethanol and cleared in xylene or 4 minutes. The images were taken through the DIALUX 20 microscope (Leica Microsystems, Wetzlar, Germany) (10 and 25x). For quantitative analysis, H&E stained cells were counted using ImageJ software (NIH, Bethesda, MD, USA). A negative binomial model has been used to model counts as a function of treatment (T0 and T14) and interaction between treatment and patients, followed by ANOVA.

Immunohistochemical (IHC) staining

For IHC staining, sample was treated as reported above. All sections were fixed in 10% formalin, embedded in paraffin, and cut into 4 μm-thick slides. The slides were dewaxed, and the endogenous peroxidase activity was blocked by treatment with 3% hydrogen peroxide solution in methanol for 20 min. Epitope retrieval was performed by treating the slides with 10 mM sodium citrate buffer (pH 6.0) and heating in a microwave oven for two times at the high power for 6 min each. Non-specific binding was prevented by blocking with normal goat serum (1:10) for 10 min. The samples were incubated with primary antibody anti-vimentin, CD4, CD8 (Roche, Ventana Medical Systems, Tucson, AZ) following the dilution suggested by the company, for 60 min at room temperature and with the appropriate secondary antibody at 37°C for 10 min. The samples were incubated with primary antibody anti-vimentin, CD4, CD8 (Roche, Ventana Medical Systems, Tucson, AZ) following the dilution suggested by the company, for 60 min at room temperature and with the appropriate secondary antibody at 37°C for 30 min. Reactive products were visualized with 3,3'-diaminobenzidene (DAB) (Sigma-Aldrich, St. Louis, MO) as the chromogen, and the slides were counterstained with haematoxylin and coverslipped. Staining was performed on the Roche Ventana Medical Systems BenchMark ULTRA automated IHC platform using the ultraView Universal DAB Detection Kit (Roche, Ventana Medical Systems, Tucson, AZ). The images were taken through the
DIALUX 20 microscope (Leica Biosystems, Wetzlar, Germany) (5, 10, 25 and 40X). For quantitative analysis, vimentin positive cells were counted using ImageJ software (NIH, Bethesda, MD, USA). A negative binomial model has been used to model counts as a function of treatment (T0 and T14) and interaction between treatment and patients, followed by ANOVA.

Results

Definition of experimental start point for correct application of Prisma® Skin on patients affected by pressure ulcers

In this investigation, seven patients affected by pressure ulcers have been selected as described in Materials and methods section. They were permanently bedded. All patients were in treatment for almost three months, in home care setting, with standard topical protocols. After slight improvements observed during the initial three months, in the following three weeks, the healing process appeared to be stationary and in absence of an evident inflammatory response. Furthermore, we found no reduction of lesion area and appearance of the wound bed sclerosis (Fig. 1a).

In order to verify this early clinical observations and to define the experimental starting point for the application of Prisma® Skin device levelling the patients’ cohort by an histological point of view, we carried out H&E staining. It is known that the process of wound repair starts from the perilesional edge in pressure ulcers of stage III [21], for that reason, a biopsy has been made closely to the lesion edge. The samples have been treated with a standard H&E staining to highlight microscopic differences. As shown in figure 1b, patients A-G presented a spread fibrosis component with a reduced presence of fibroblasts or other cell types including cells of the inflammatory response (Fig. 1b panels a-f). A similar condition has been also revealed for patient G, where, beyond a moderate inflammation suggested by the presence of inflammatory infiltrates, we found fibroblasts concentrated in one side of the section (Fig. 1b, panel g, yellow arrow). We proved this aspect also below.

The wound bed sclerosis is confirmed by the absence of CD4 and CD8 lymphocytes

We next evaluated by IHC the inflammatory state of the patients’ wound beds, focusing on the presence of the lymphocyte population as the most important cell components of immune system, The figure 2 shows samples from patients A-G stained with CD4 and CD8. We found low levels of both T4 lymphocytes (panels a-g), and T8 cells (panels h-n). Just for the patient B few T8 lymphocytes are concentrated only in a part of the section (panel i, yellow arrow).

The treatment with Prisma® Skin enhanced cell recruitment in the wound areas

As mentioned above, we assumed the T0 time as experimental start point for the correct application of the Prisma® Skin device. To avoid the risk of the appearance of general and local complications and to have a good clinical range of monitoring time useful for all the clinical and histological evaluations of cellular activity, we concentrated on a time range 0-14 days (To-T14).

The patients were treated for 14 days with Prisma® Skin, for the management of pressure ulcers, according to the principles of the Wound Bed Preparation.

Skin biopsies from patients A-G were collected at T14 as previously described for T0 samples and therefore they were stained with H&E. Differently from T0 samples, histological analysis performed as reported in Materials and methods section, showed a relevant cell architecture (Fig. 3a, panels a-g). This result confirmed that the treatment with Prisma® Skin significantly induced the recruitment of cells in wound areas.

Prisma® Skin was able to specifically engage fibroblasts in the wound area

We performed further IHC analysis with anti-vimentin antibody to define the presence of fibroblasts at T14 of patients’ biopsies. Differently from the samples shown at T0 (Fig.4a, panels a-g), after the treatment with Prisma® Skin we found a significant amount of vimentin signal confirming the presence of fibroblasts (Fig. 4b, panels h-n for patients A-G). In figure 4c it is shown a photography reportage of the patients and their related wound diameter at T0 and T14, after treatment with Prisma® Skin. These images confirmed the modification of the wound bed which started to lose its sclerotic features, as a fundamental phase to
trigger the following wound closure which did not verify in 14 days of treatment.

Discussion

Pressure ulcers and common chronic skin lesions represent one of the most expensive health performances: about the 3% of the worldwide health spending is addressed to the therapies of these diseases. Thus, the knowledge of the etiopathogenetic mechanisms and the staging are discriminating factors to design an appropriate treatment plan. Indeed, for our analysis, we firstly defined the T0 as starting point for the application of Prisma® Skin through the evaluation of tissue architecture. Furthermore, the new concept of topical treatment of skin lesions considers the dressings as an instrument able to activate solver phenomena and to remove the impediments for the healing. The aim of this kind of dressings is to enhance the re-epithelisation, rather than the cicatrisation in order to regenerate viable tissue. Therefore, this case series answered to the need to identify efficient treatments based on pharmaceutical devices with new active substances. Particularly, we evaluated the clinical effects of the mesoglycan containing pharmaceutical device Prisma® Skin on patients with pressure ulcers, focusing on fibroblasts activity.

The mesoglycan has been used, in general therapy, in the treatment of vascular diseases also associated to chronic ulcers [5-8]. Our recent in vitro investigations showed that Prisma® Skin is able to enhance re-epithelialization and granulation processes, acting on human epidermal keratinocytes and dermal fibroblasts [12-17].

Therefore, it was necessary to define a clinical in vivo condition to evaluate the effects of fibroblasts by Prisma® Skin and confirm our previous in vitro results. The definition of the experimental starting point (T0) has become an important aspect for our study since both clinical and histological evaluations were required to avoid the generation of false positive results about the pro-activator and pro-migratory functions of the device.

The etiopathogenesis of pressure ulcers, compared to other types of chronic skin lesions, is very simple and univocal. This allowed us to select seven patients affected by lesions attributable to the classical pressure ones for region (sacral), stage (III) and status (cleansed and/or tending towards wound bed).

In order to have a good clinical range of monitoring time, useful for all the clinical and histological evaluations of fibroblasts activity we concentrated on a time range 0-14 days.

The H&E and IHC staining of patients’ biopsies at T0 proved the lack of cell structure in the analyzed tissues with a tendency to wound bed sclerosis, and reduced inflammatory activity. In fact, we found a low presence of lymphocytes T CD4 and CD8. In only two cases this situation did not occur and we eliminated the patients from the study to avoid any initial inconsistency.

As previously stated, at T0 the inflammatory activity appears reduced, consequently fibroblasts are not recruited probably because of a diminished concentration of growth factors deriving from reduction of cell migration. In this scenario, GAGs and PGs could not be produced with reduced angiogenesis and cell proliferation. Overall, this information showed that all the activities related to tissue repair progressively arrest, probably because the lack of the inflammatory response leads the system to consider repaired the lesion.

Notable, fibroblasts produce collagen and PGs during several weeks after the injury, in parallel the endothelial cells form capillary structures and synthesize GAGs, above all HA, CS and DS, able to modify the overall concentration of these macromolecules in the wound area [22]. HA is the most abundant for the first two weeks and is followed by DS and CS [23]. When cell proliferation rate reaches a plateau, the levels of HS increase and allow the HS-PG2 to regulate the tissue repair through the induction of keratinocytes activation and angiogenesis [24].

Our data show at T14, after treatment with Prisma® Skin, not a relevant wound closure but a significant number of migrated fibroblasts and these cells are further orientated in the area, probably because they are ready to differentiate in myofibroblasts, as we have previously observed in vitro [12, 25]. Generally, during the phases of wound healing repair, fibroblasts and other mesenchymal cells penetrate the inflammatory side of the lesion in response to specific growth factors [26].
molecular aspect underlining our results could be explained through the ability of CS and DS to support the polymerization of collagen chain and interfere with the production of growth factors and of the pro-inflammatory nitric oxide. Furthermore, HS is able to anchor to the surrounding ECM and release Interleukin (IL)-1, IL-6, prostaglandin (PG)-E2 and TGF-β with pro-angiogenesis effects [27, 28]. Finally, HS and HEP are capable to protect the basic Fibroblast Growth Factor (bFGF) by the inactivation for proteolysis, heat, acids and not-enzymatic glycosylation and to stabilize the interaction between FGF and its receptor FGFR [29, 30].

Although we did not attend to a significant wound closure, our findings suggest that the Prisma® Skin could be able to induce an increase in proliferative activity, confirming by clinical evaluation that showed a recovery of cell proliferative activity with the formation of granulation tissue.

In conclusion, for the first time, the device Prisma® Skin, mainly containing mesoglycan, has been applied to patients’ pressure ulcers. This analysis began the translation in clinical settings what we have previously seen in vitro. Particularly, our results confirm the pro-proliferative, migratory, invasive activities of the mesoglycan on fibroblasts, one of the most important cell population involved in tissue repair. This data highlights as cell recruitment can be considered a fundamental initial phase of tissue repair to obtain the following wound closure.

Further studies will be performed to assess the efficacy of the device on a larger number of patients and for a longer range of time. Moreover, other investigations are needed to clarify the mechanism underlying Prisma® Skin effects.

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Competing financial interests
The authors declare no competing financial interests.

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Figure 1. pictures showing a bedsore wound (a) and tissue sections stained through H&E (panels a-g). (b) at T0.
Figure 2. IHC staining for patients’ tissue sections for CD4 and CD8 at T0. Magnification 25x. Bar= 100µm.
Figure 3. (a) tissue sections stained through H&E. The sections have been harvested at T0 and T14 (panels a-g). Black bar= 50µm, magnification 25x; yellow bar= 200 µm magnification 10x. (b) The number of cells per µm² was counted in at least three fields from three different specimens for each patient. In the graph the average value is shown for patients at T0 (blue dots) and T14 (red square). A negative binomial model was used to model counts as a function of treatment (T0 and T14) and interaction between treatment and patients, followed by ANOVA. P value was < 2.2e-16 for treatment and < 2.2e-16 for the interaction treatment-patient.
Figure 4. (a) IHC staining for tissue sections of patients A-G, for vimentin at T0 and T14. Magnification 25x. Bar= 100µm. (b) The number of cells positive for vimentin per µm² was counted in at least three fields from three different specimens for each patient. In the graph the average value is shown for patients at T0 (blue dots) and T14 (red square). A negative binomial model was used to model counts as a function of treatment (T0 and T14) and interaction between treatment and patients, followed by ANOVA. P value was < 2.2e-16 for treatment and < 2.2e-16 for the interaction treatment-patient. (c) Pictures showing a bedsore wound at T0 and T14, after the treatment with Prisma® Skin.