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Development of a novel beta-glucan supplemented hydrogel spray formulation and wound healing efficacy in a *db/db* diabetic mouse model

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Abstract

To relief the severe economic and social burdens and patient suffering caused by the increasing incidence of chronic wounds, more effective treatments are urgently needed. In this study, we focused on developing a novel sprayable wound dressing with the active ingredient β-1,3/1,6-glucan (βG) ~~isolated from baker's yeast. , and applied as a semisolid hydrogel formulation is~~ Since βG is already available as the active ingredient in a commercial wound healing product provided as a hydrogel in a tube (βG-Gel), the ~~that has been shown to increase the rate of wound closure in both diabetic mice and patients. The objective of this work was to develop a sprayable βG formulation that could~~sprayable format should bring clinical benefit by being easily sprayed onto wounds; whilst retaining ~~its~~ βG-Gel's physical stability, biological safety and wound healing efficacy. ~~Fifteen different p~~Potentially sprayable βG hydrogels were ~~-~~ therefore formulated, based on an experimental design setup. O~~and their rheological properties examined~~ne ~~. Based on its physical characteristics, one formulation (βG-s~~Spray formulation, named βG-Spray,~~),~~ was selected ~~selected~~ for further investigation, as it showed favorable rheological and spraying properties~~for further investigation~~. ~~These investigations included assessment of fluid affinity (absorption and donation), cytotoxicity *in vitro*, wound healing efficacy i*n vivo* and formulation stability.~~ The βG-Spray was furthermore found to be stable at room temperature for more than a year, retaining its rheological properties and sprayability~~for more than a year, ~~. The ~~Importantly, the formulation was sprayable using a commercially available spray system. non~~-cytotoxicity of βG-Spray in keratinocytes *in vitro*, was shown to be promising even at the highest tested concentration of 100 µg/ml~~and aseffective as the βG-Gel in terms of its ability to promote wound healing in healing-impaired animals~~. The ~~The formulated~~ βG-Spray also displayed favorable fluid affinity characteristics, with a capacity to both donate and absorb close to 10% fluid relative to its own weight ~~similar to those of βG-Gel~~. Finally, t~~Importantly, the~~

formulation was sprayable using a commercially available spray system. he βG-Spray was proven comparably effective to the commercial product, βG-Gel, and superior to both the water and the carrier controls (NoβG-Spray), in terms of its ability to promote wound healing in healing-impaired animals. Contraction was found to be the main wound closure mechanism responsible for the improvement seen in the βG-treatment groups (βG-Spray and βG-Gel). In conclusion, encouraging results support further investigation of βG-Spray the novel sprayable βG formulation, confirmed its potential to expand the clinical use of βG as wound dressing in the clinical setting.

Key words: beta-glucan; hydrogel; spray formulation; *db/db* diabetic mice; wound dressing; wound healing, chronic wounds

Abbreviations:

βG = β-1,3/1,6-glucan

βG-Gel = The commercially available semisolid hydrogel formulation containing 2 % βG

βG-Spray = Spray formulation containing 2 % βG

CMC = Sodium carboxymethyl cellulose

db/db mouse = mouse model of type 2 diabetes mellitus

HaCaT = Human adherent keratinocytes

H&E = Haematoxylin & Eosin

HPMC = Hydroxypropyl methylcellulose

MTT = Colorimetric assay cell proliferation kit I

NoβG-Spray = Spray formulation without βG

PDFG-BB = rh-platelet-derived growth factor-BB

TGF-α = rh-transforming growth factor-α

1. Introduction

The impact of chronic wounds on society is ~~both~~ immense, but ~~and at the same time~~ difficult to quantify [1,2], chronic wounds are ~~, which otherwise is known to~~ severely lowering the patients quality of life ~~of patients~~ [3–5]. With both an aging population, and ~~with~~ the prevalence of diabetes expected to rise dramatically in the coming years [6], ~~it is anticipated that~~ the prevalence of chronic wounds in general, and diabetic foot ulcers in particular, ~~will also~~are expected to rise. ~~The knowledge that~~Taking into account that there is approximately a one-third chance ~~of~~ that people having diabetics also develop foot ulcers ~~over the course of their lifetime is of great concern~~ [7], and knowing that ~~. As~~ chronic wounds already represent the largest contributor to the annual cost of wound treatment [2], it is essential that ~~effective~~ cost-efficient therapies are developed ~~to reduce the financial burden of this clinical problem. The development of novel, and more effective, wound healing therapies will hopefully give rise to improved treatment outcomes~~ [8–10]. The wound healing process involves various cell types and signalling molecules that sequentially coordinate the different phases of the wound repair processes, namely: hemostasis, inflammation, proliferation and ~~remodeling~~remodelling. In chronic wounds, the healing process stalls in the inflammatory phase, which has been attributed to a range of pathophysiological defects, including impaired macrophage function [11,12]. ~~That being the case, wound therapiesdesigned to actively encourage the resolution of inflammation, and facilitate the progression of wound healing, are required to address the ever-growing issue of chronic wounds~~Understanding the underlying pathology and healing status of a wound is important in selecting the most appropriate wound ~~healing product~~dressing, as there is no universally effective wound product [13]~~.~~

Academia and industry are now focusing more on developing advanced and active wound healing products, by developing specialized products for different wound-types [5]. Advanced

wound dressings can either influence the healing processes directly, or indirectly, by the release of bioactive substances within the wound [14–17].

β-glucans are carbohydrate polymers that are found in the cell walls of many organisms, including yeast, fungi and certain bacteria. Throughout evolution, the mammalian immune system has learned to identify these structures as Pathogen Associated Molecular Patterns (PAMPs), and β-glucans are by this mechanism ~~known to~~ induc~~e~~inge immune modulatory effects in humans [18–22]. β-glucans have been shown to ~~be capable of revert~~reverting immuno-compromised macrophages back to a functioning phenotype in humans, an effect that may explain the benefit of β-glucan as an active ingredient in the treatment of chronic wounds [23–27]. Another reported benefit of β-glucans is their ability to modulate the wound healing process, and reduce scarring in mice, which may prove beneficial to patients with excessive and disfiguring scarring [28].

~~Academia and industry are now focusing more on developing advanced and active wound healing products to combat the chronic wound pandemic, by developing specialized products for different wound-types [5]. As opposed to traditional/passive dressings, advanced wound dressings incorporate agents that can either influence the healing processes directly, or indirectly, by encouraging the release of bioactive substances within the wound [24–27]. Understanding the underlying pathology and healing status of a wound is important in selecting the most appropriate wound healing product, as there is no universally effective wound product [28].~~ β-1,3/1,6-glucan (βG) from baker's yeast (*Saccharomyces cerevisiae*), has previously been proven to have favorable effects on wound healing, both in the format of electrospun nanofibers [27], and as a hydrogel [23]. At present, commercially available βG~~β-glucan~~-products for chronic wound treatments are formulated ~~in~~ as semisolid hydrogels (e.g.

Woulgan®, Biotec BetaGlucans AS, Norway), ~~that are~~ applied to the wound with a gloved finger. Hydrogels are moisture retentive products that are recommended for use on dry to low exuding deep chronic wounds, and ~~are~~ known to alleviate chronic wound pain.

Based on the reported therapeutic advantages of spray administration for topical wound treatments, this method of application may be an appropriate method by which to deliver βG ~~β-glucan hydrogel formulations~~ to chronic wounds [29–31]. Spray administration is a simple, non-contact method, which permits quick and easy application/re-application of liquid/semi-solid formulations to wounds [32]. The non-contact nature of spray administration makes it particularly attractive for the treatment of painful wounds. But, since we failed in a previous attempt to prepare a sprayable wound dressing with βG as the active ingredient, due to adverse effect seen for the formulations during *in vivo* testing in mice [33], an alternative and more effective βG-Spray formulations was targeted. Since these previously detected adverse effects were found to be related to the applied thickening agent, Carbopol, we ~~The~~ aimed ~~of this work was~~ to develop a sprayable ~~βG~~ βG-formulation using, instead of Carbopol, a medium viscosity carboxymethyl cellulose (CMC) as a thickening agent, and glycerol as a humectant~~;~~. B~~b~~oth CMC and glycerol ~~of which~~ are extensively used in wound healing products and have well-documented effects on wound healing [5,34–36]~~. The active ingredient selected for this study was β-1,3/1,6-glucan (βG) from baker's yeast (*Saccharomyces cerevisiae*). The same active ingredient (βG) has previously been proven to have favorable effects on wound healing when formulated in an electrospun nanofiber format, intended for medium to high exuding wounds [22]. Also, in a randomized double-blind diabetic foot ulcer study, the same active ingredient formulated as a 2.0 % (w/v) βG-gel in water, was found to promote wound closure [18]~~. Our reference formulation, βG-Gel (comprised of 2.0 % βG w/v, a high viscosity CMC and glycerol), is a commercially available

wound healing product, with well documented ~~and has been shown to significantly improve healing rates in~~ effects in chronic wounds of different etiology [26,37]. The composition of this non-sprayable reference formulation thus also encourage to apply CMC and glycerol in the development of the novel βG sprayable formulation.

In this study, the rheological properties of the spray formulation (βG-Spray) and a carrier control spray (NoβG-Spray), including stability and sprayability were initially established. Secondly, toxicity to immortalized human keratinocytes and fluid affinity were investigated and compared to the well-characterised commercially available β-glucan hydrogel (βG-Gel). Finally, the spray formulation, βG-Spray, was~~the carrier control spray (NoβG-Spray) and the aforementioned commercially available β-glucan hydrogel (βG-Gel), were~~ evaluated ~~and compared~~ in terms of ~~their~~ its impact on the healing of full-thickness excisional wounds in the healing-impaired *db/db* mouse model, together with NoβG-Spray and βG-Gel, for comparison, and water and growth factors (PDGF-BB and TGF-α), as negative and positive control, respectively.

2. Materials and methods

2.1 Materials

Glycerol (1, 2, 3-propanetriol) was purchased from VWR (Fontenay sous Bois, France). Milli-Q water was produced using a Direct 8 Water Purification System by Merck Millipore (Billerica, MA, USA). Soluble β-1,3/1,6-glucan (βG; 2.5 % w/w) and Woulgan® Gel (βG-Gel) were gifted by Biotec Betaglucans AS (Tromsø, Norway). Sodium carboxymethyl cellulose (CMC) 7M1F (MW 250,000) was purchased from Ashland (Wilmington, DE, USA). Gelatin from porcine skin was obtained from Sigma-Aldrich (Taufkirchen, Germany) and Acto™ Agar was purchased from BD (Le Pont de Claix, France). The HaCaT cell line (immortalized human keratinocytes) was purchased from Thermo Fisher Scientific (Waltham,

USA). RPMI growth medium was obtained from Sigma Aldrich (Steinheim, Germany). The MTT cell proliferation kit assay was purchased from Roche (Sigma Aldrich). The rh-platelet-derived growth factor-BB (PDGF-BB) and rh-transforming growth factor-α (TGF-α) were purchased from PeproTech EC Ltd (London, UK). Isoflurane (IsoFlo®) was from Zoetis (London, UK), and Buprenorphine (Vetergesic®) was purchased from Alstoe Animal Health (Espoo, Finland). 10% Neutral Buffered Formalin, Haematoxylin and Eosin were purchased from Sigma. Picrosirius red solution was purchased from Pioneer Research Chemicals (UK).

2.2 Preparation of the spray formulations

The formulations were prepared from four ingredients; CMC, glycerol and water, and the active ingredient βG. βG was provided as a sterile hydrogel with 2.5 % (w/w) soluble β-1,3/1,6-glucan dispersed in water, prepared by a patented method [38]. This βG-hydrogel can be liquefied by heating, and contains soluble βG with a MW of around 7 x 10^5 g/mol. ~~by first~~ The first step of the preparation was to disperse and wet~~ing~~ CMC in glycerol, before further dispersion in ~~adding~~ Milli-Q water ~~and~~ followed by addition of ~~a~~ preheated (50 °C) ~~2.5 % (w/w)~~ βG ~~2.5 % (w/w) (50 °C)~~. All of the respective ingredients were adjusted to reach the aimed concentrations. ~~.~~ The composition variables/weight ratio applied for the different ingredients are ~~in the study are~~ given in the Supplementary Table S1. All ingredients ~~. which~~ were thoroughly mixed using an Ultra-Turrax (T25, IKA®-Werke GmbH & Co. KG, Germany). The formulations were autoclaved at 121 °C for 20 min and allowed to swell for a minimum of one week at room temperature, before further testing. For the initial spray test, 15 formulations were prepared, with concentrations of βG ranging from 1.6 to 2.4 % (w/w), CMC from 0.5 to 2.5 % (w/w) and glycerol from zero to 20 % (w/w). ~~The composition variables applied in the study are given in Supplementary.~~ The design matrix was obtained by Design-Expert® software (version 10.0.8.0) from Stat-Ease, Inc. (Minneapolis, MN. USA). The design was a full two-level factorial design with 3 factors (2^3 = 8 combinations) with 4

four center points. The factorial points were replicated to give a total of 20 runs representing ~~8~~ eight different formulations. The design was augmented with an additional block of axial star points and two additional center points to make a central composite design, giving a total of 34 runs representing 15 different formulations [39].

2.3 Sprayability

Spraying characteristics were tested using two versions of an airless spray nozzle Comfort®-actuator (Ursatec Verpackung GmbH, Germany) delivering either 45 or 140 µL per dose, attached to a 10 ml polypropylene-container. The run order was randomly conducted, assorted by Design-Expert® to exclude bias. The actuators were placed 10 cm from a horizontal oriented sheet of paper, pressed and the sprayability recorded based on the observation made.

2.4 Rheological assessments

The rheological properties of the 15 different formulations, including the selected βG containing spray formulation (βG-Spray), a carrier control (NoβG-Spray) and a marketed βG gel (βG-Gel), were investigated using a Discovery HR-2 Hybrid Rheometer (TA Instruments, New Castle, DE, USA), equipped with Peltier plate temperature control and a 40 mm parallel plate geometry. Samples were carefully loaded on to the Peltier plate using a spoon to prevent any "pre-shear". The geometry was lowered to a gap of 1050 µm (trim gap), excess gel was removed, and the plate ~~was~~ lowered to a 1000 µm gap (geometry gap). A "temperature soak step" of minimum 1 min at 25 °C was included prior to all measurements. ~~.~~ An **"**oscillation time sweep protocol" and an "oscillation amplitude sweep protocol" were run in succession on each sample. The "oscillation time sweep protocol" was used to measure the elastic modulus (G′), viscous modulus (G″) and phase angle (δ; $tan\delta = \frac{G''}{G'}$) of the unbroken gel (measured within the linear viscoelastic range), while the "oscillation amplitude sweep protocol" was used to determine the yield stress. The yield stress equals the oscillation stress required to "break" the gel, defined here as the modulus crossover (G″= G′) when the

formulation loses its elastic dominant properties. The "oscillation time sweep protocol" was carried out using a displacement of 0.001 rad at 1.0 Hz over 60 s, while the "oscillation amplitude sweep protocol" used a torque increment per step of 100 μN·m from 100 to 10 000 μN·m with an oscillation frequency of 1 Hz. An "oscillation temperature ramp protocol" was used to measure the melting (gel-to-sol) temperatures of the formulations. For the temperature ramps, the geometry was also fitted with a solvent trap to prevent moisture from evaporating. This protocol was run with a displacement of 0.001 rad at 1.0 Hz with the following temperature program: 180 s at 25 °C; 1.0 °C/min ramp up to 55 °C. The melting temperature (gel-to-sol) was defined as the temperature of modulus crossover in the increasing temperature ramp.

2.5 Stability

In order to test the stability of the formulations selected for the *in vivo* experiment (βG-Spray and NoβG-Spray), the "oscillation time sweep protocol" and "oscillation amplitude sweep protocol" were applied as previously described. The formulations were stored at room temperature, and measurements conducted after 1, 2, 6, 14, 26 and 56 weeks storage. All results were processed ~~by~~ using the Trios software v. 3.2.0.3877 (TA Instruments, New Castle, DE, USA).

2.6 Fluid affinity

Fluid absorption and donation were tested according to the EU industry standard EN 13726–1:2002, as previously reported by our group [33,40]. We used a simulated wound exudate (Solution A), emulating the ion concentration of human serum or wound exudate (142 mmol Na^+, 2.5 mmol Ca^{2+}). First, 60 mL syringes (B. Braun Melsungen, Hessen, Germany) with the tip removed, were filled with 10.0 ± 0.1 g gelatin or the same amount of agar solution. Thereafter, the syringes were covered with Parafilm® and left to settle for 3 h at 25 ± 2 °C. After removing the Parafilm®, the total weight (W_1) of the syringe with its content was

recorded. Thereafter, 10.0 ± 0.1 g of test formulation was added to the syringe and the total mass (W_2) (corresponding to W_1 + test formulation), was recorded. The syringe was then again covered with Parafilm® and incubated at 25 ± 2 °C for 48 h. After incubation, the Parafilm® was removed and the mass was recorded (W_3) before removing the test formulation. Finally, the mass of the syringe with either the formulation-exposed agar or gelatin (W_4) was recorded. The fluid donation or absorption (% w/w) of the formulation (W_5) was calculated using the equation (Eq. 1, below), also described in the EU industry standard EN 13726–1:2002 [40]. Five replicate experiments were performed on each formulation.

(Eq. 1)

$$W_5 = \left(\frac{(W_3 - W_4) - (W_2 - W_1)}{(W_2 - W_1)}\right) x\ 100\ \%$$

2.7 Cytotoxicity of spray formulations

The cytotoxicity of the spray formulations was tested *in vitro* using human keratinocytes (HaCaT cells), as reported previously by our group [27]. In short, the cells (1 x 10^5 cells/mL) were cultured in flat bottomed 96 well plates containing 90 µL/well of culture medium supplemented with 10 µL of ~~neat~~ growth media (for control) or media containing test samples to give final exposure concentrations of 1, 10 or 100 µg/mL. After incubation for 24 hours, 10 µL of MTT was added to all wells, and the plates incubated for a further 4 hours. After adding 100 µL of a solubilizing reagent, the cells were incubated for another 24 hours. An ELISA plate reader was used to detect the 580 nm UV absorption of soluble formazan. The UV absorption for the control group was used to normalize the data, with the control ~~taken~~ set to be 100 % viable. The effects of the test samples at various concentration on cell toxicity were expressed as mean percentage viability of two independent experiments for each sample. Control samples were tested in quadruplicate.

2.8 Effect of spray formulations on wound healing in diabetic mice

In vivo evaluation of the wound healing potential of the formulations was undertaken in the healing-impaired *db/db* diabetic mouse model, according to the methods previously described by our group [27,33]. The experiment was conducted in accordance with the specific requirements of diabetic animals and in agreement with -UK Home Office regulations [41]. Nine- to ten-week-old male *db/db* diabetic mice (BKS.Cg-m Dock7^{m} +/+ Lepr^{db} /J mice) purchased from Jackson Labs (Bar Harbor, ME, USA), were allowed to acclimate in the animal house facility for one week prior to the start of the study. Fifty animals (weight 45.3 ± 2.8 g) were randomly allocated to five groups (10 mice per group): i) positive control (10 µg rh-platelet-derived growth factor-BB [PDGF-BB] and 1 µg rh-transforming growth factor-α [TGF-α] (PeproTech EC Ltd, London, UK) in 0.5 % w/v HPMC (Sigma, UK); ii) βG-Gel (commercial product), iii) βG-Spray, iv) NoβG-Spray (vehicle control) and, v) negative control (sterile water for injection).

Full-thickness, 10 x 10 mm square wounds were created approximately 10 mm from the spine on the left mid dorsal flank using straight iris scissors. The area was cleansed and shaved before wounding and covered with Bioclusive® film dressing (Systagenix Wound Management, Gargrave, UK) after wounding. Treatment formulations were injected through the film dressing (into the wound) using a 27-gauge needle. The administered dose was 50 µL for all treatments. The three test formulations and the negative control (water) were applied on post-wounding days 0, 2, 4, 6 and 8; whereas the positive control was applied on post-wounding days 0, 1, 2, 3, 4, 5 and 6 (detailed description in Supplementary Table S2, in Appendix 1).

Anesthetisia was induced using 4 % isoflurane/air (IsoFlo®, Zoetis, London, UK) and maintained with 2 % isoflurane. Analgesia, in the form of Buprenorphine (Vetergesic®,

Alstoe Animal Health, Espoo, Finland), was administered (75 µg/kg, s.c.) to reduce discomfort immediately after wounding, and subsequently according to clinical need.

2.8.1 Macroscopic assessment of wound healing

The "open wound area" (A_{GT}) and the "extent of contraction" (C_{GT}) of each wound were measured (using Image Pro Plus image analysis software - version 4.1.0.0, Media Cybernetics, Rockville, MD, USA) from calibrated digital wound photographs (Fig. 1) taken on post-wounding days 0, 4, 8, 12, 16, 20 and 24. The "original wound area" (A_0) was 10 x 10 mm. "Percentage wound closure" over time (relative to the original wound area), and the contribution of "wound contraction" and "wound re-epithelialization" to "wound closure", were derived from these measures (according to Equations 2, 3 and 4, below).

(Eq. 2) "Percentage wound area remaining": The open wound area remaining at a given time point relative to the original wound area.

$$\left(\frac{A_{GT}}{A_0}\right) x\ 100\ \%$$

(Eq. 3) "Percentage wound contraction": The difference between the contracted wound area at a given time point and the original wound area, as a percentage of the original wound area.

$$\left(\frac{A_0 - C_{GT})}{A_0}\right) x\ 100\ \%$$

(Eq. 4) "Percentage re-epithelialization": The contracted wound area at a given time minus the open wound area at that given time, as a percentage of original wound area.

$$\left(\frac{C_{GT} - A_{GT}}{A_0}\right) x\ 100\ \%$$

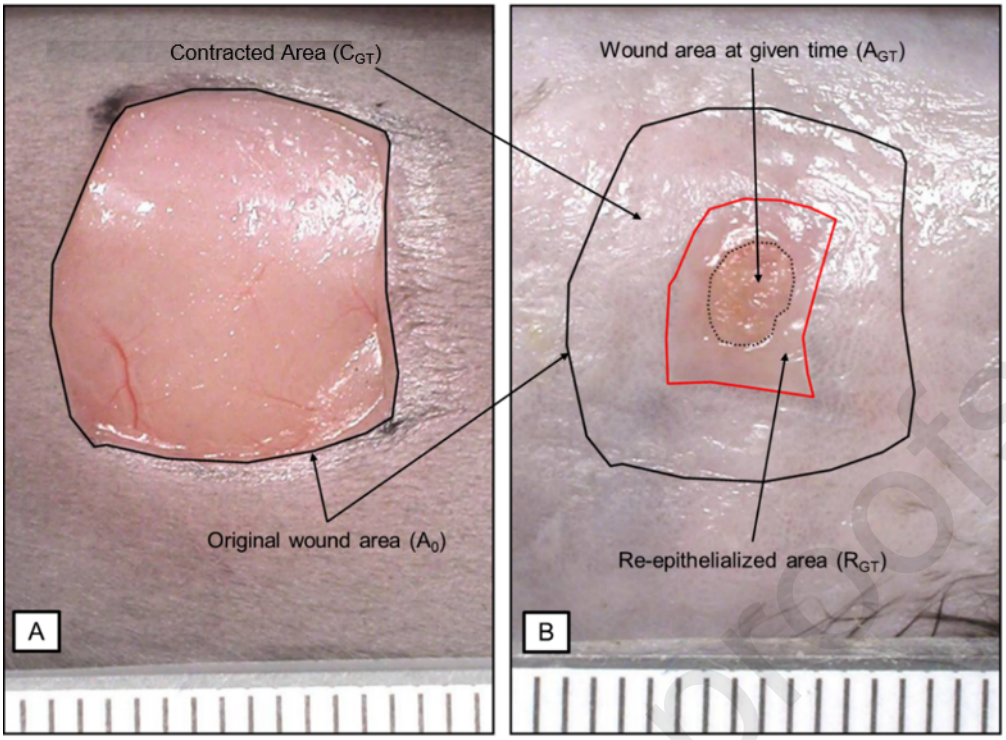

Figure 1. Illustration of the wound healing parameters and terminology used to assess the progress of wound closure during the study. A) A wound on day 0 (day of surgery), B) The same wound on post-wounding day 12.

2.8.2 Histologic assessment of wound healing

Skin samples, containing the wound with surrounding normal skin, were harvested from four animals in each treatment group on post-wounding day 24. These t~~T~~issue samples were fixed (10% Neutral Buffered Formalin, Sigma) and processed to paraffin wax. Sections (6 µm), taken through the center of each wound, were stained with: i) Haematoxylin & Eosin (H&E) and ii) the collagen-specific stain Picrosirius Red [42]. The stained sections were then digitally scanned (at x20 equivalent magnification) using an Aperio AT2 whole slide scanner (Leica Biosystems, Germany). "Granulation tissue depth" and the "extent of wound re-epithelialization" were measured from digital scans of H&E-stained sections using Aperio Imagescope software (version 12.3.0.5056, Leica Biosystems, Germany). "Granulation tissue

depth" (d) was measured at ~~9~~ nine equally-spaced points across each wound and a mean depth calculated for each wound. The "amount of new epithelium" extending from the two wound edges (A and B, Fig. 2)), was expressed as a percentage of the full length of the wound (A+B+C). "Granulation tissue depth" and "% re-epithelialization" (calculated as described in Eq. 5) were compared between treatment groups.

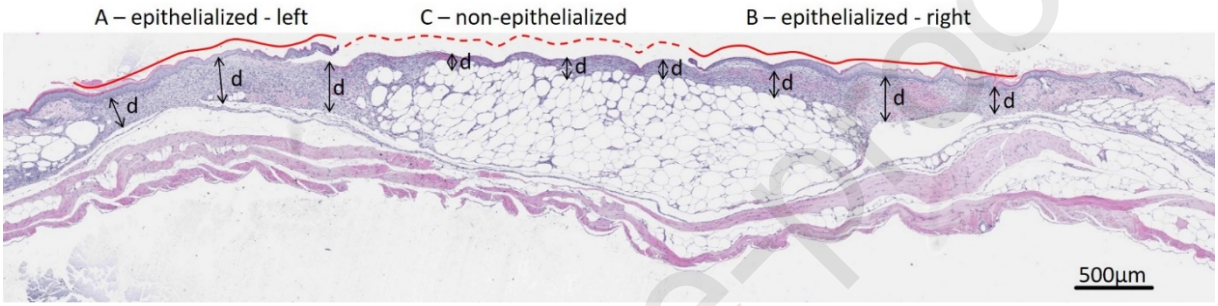

Figure 2. Post-wounding day 24 diabetic mouse wound section stained with haematoxylin and eosin (H&E) showing: "re-epithelialization from the wound margins" (A & B), a "central non-epithelialized region" (C), and "granulation tissue depth" (d) at ~~9~~ nine points across the wound.

(Eq. 5) Percentage re-epithelialization:

$$\left(\frac{A+B}{A+B+C}\right)x\ 100\ \%$$

"Collagen deposition" within wound tissues was quantified from Picrosirius Red-stained sections. Digital scans were viewed using Image-J software (NIH, USA) and three regions of interest; left margin, central wound and right margin (each 1000 x 1000 µm) were identified. Each region of interest was then extracted and viewed using Image Pro Plus software (Fig. 3A and 3B), and images manipulated (using a ~~preset~~pre-set threshold) to exclude all non-

collagenous structures (Fig. 3C). The area within each region of interest "occupied by collagen" (i.e., red staining) was measured and expressed as a percentage of the whole region of interest (i.e., 1 mm^2). The "collagen content" of the two outer (wound marginal) regions of interest was averaged, and "collagen deposition" with the central and marginal regions was compared between treatment groups.

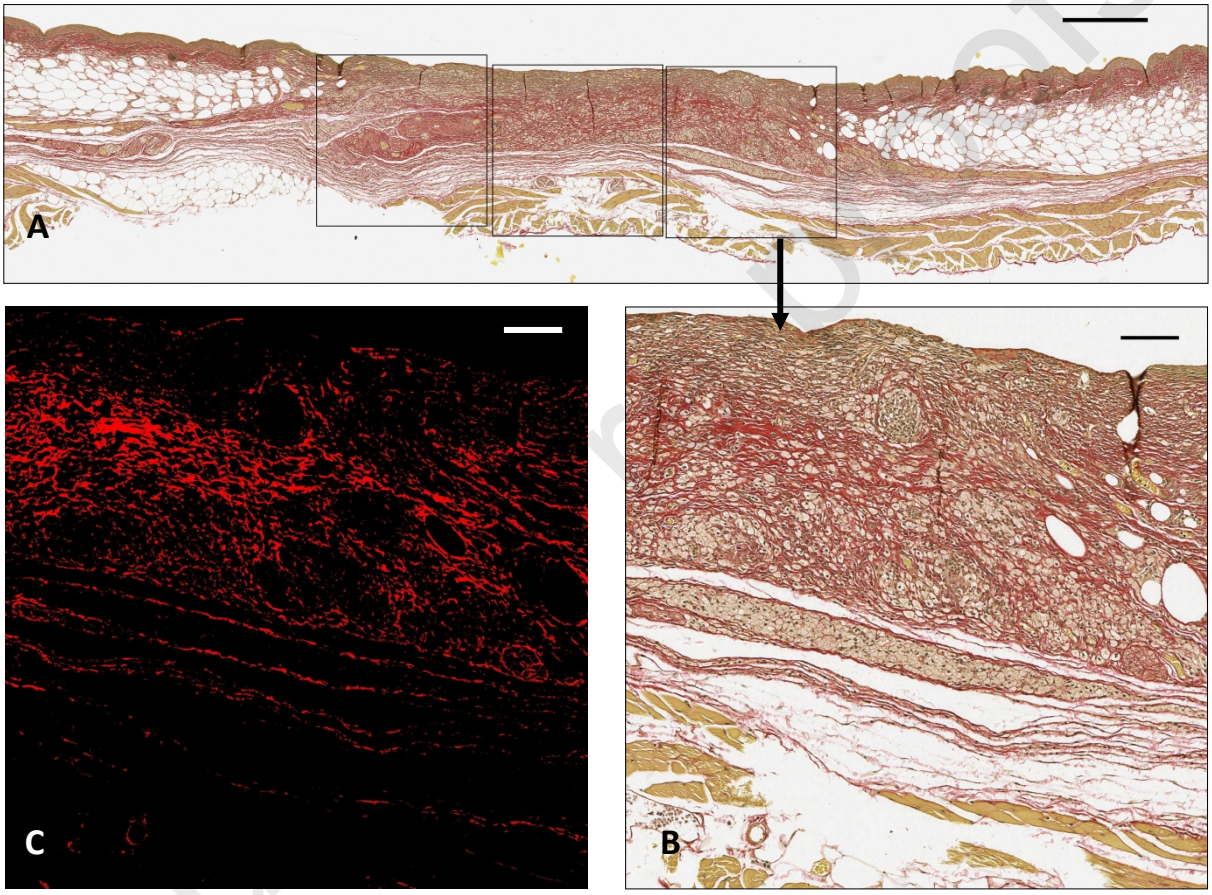

Figure 3. A) Diabetic mouse wound section (day 24) stained specifically for collagen using with Picrosirius Red, with 3 (1000 ~~µm~~ x 1000 µm) sub-regions shown (bar 500 µm); B) enlarged right-marginal region - dark red staining is mature collagen (bar 100 µm); C) Image B manipulated to display collagen staining only (bar 100 µm).

2.9 Statistical Analysis

For stability and the fluid affinity data, outliers were removed using maximum normed residual test [43]. For the stability study, a two-tailed t-test was used to determine the difference between time points, and p values <0.05 were considered significant. In the *in vivo* study, the two sample non-parametric statistical test Mann-Whitney U-test was used to test for statistically significant differences between groups, with a significance level of 5 % ($p<0.05$).

3. Results

3.1 Sprayability and rheological assessments, including stability

The nozzle Comfort® spray system from Ursatec Verpackung GmbH, Germany, was selected for the study. It is ~~constructed~~ produced for multiple use without the need for preservatives, as an inner bag collapses as the container empties (Fig. S1, Appendix 1). Actuators giving both 45 and 140 µL per dose were tested. All the tested βG-containing spray formulations ~~prepared~~ (Table 1) were sprayable with both ~~the 45 and 140 µL per dose~~ actuators, and the formulations were ~~with a spray distance of 10 cm, and had a~~ spread on an area of approx. 5 cm ø at a distance of 10 cm from the actuator.

The results from the rheological measurements ~~are summarized in Table 1~~, including ~~. We determined~~ the respective formulations` phase angle, yield ~~point~~ stress and melting point (gel-to-sol) ~~of the different spray formulations and βG-Gel.~~, are summarized in Table 1.

Table 1. Rheological characteristics of the β-glucan containing formulations. All measurements are an average of two independent experiments except for the center points (± SD).

Formulation no				Oscillation time sweep	Oscillation amplitude sweep	Oscillation temp. ramp
	βG conc. % (w/w)	CMC conc. % (w/w)	Glycerol conc. % (w/w)	Phase angle, δ (degree)	Yield stress (Pa)	Melting point (gel-to-sol) (°C)
1	1.6	1.5	10	9.17 (± 0.6)	44.0 (± 0.9)	38.9 (± 0.4)
2	1.8	1.0	5.0	6.75 (± 1.6)	29.6 (± 5.0)	38.6 (± 0.1)
3	1.8	1.0	15	6.70 (± 0.4)	30.2 (± 5.6)	42.6 (± 0.0)
4	1.8	2.0	5.0	10.8 (± 0.1)	52.2 (± 3.3)	37.8 (± 0.1)
5	1.8	2.0	15	9.95 (± 0.4)	68.4 (± 1.3)	41.7 (± 0.1)
6	2.0	0.5	10	5.57 (± 0.1)	31.5 (± 4.7)	40.9 (± 0.6)
7	2.0	1.5	0.0	9.61 (± 0.9)	39.4 (± 6.2)	34.6 (± 0.4)
8[d]	2.0	1.5	10	7.08 (± 1.2)	62.5 (± 7.5)	40.7 (± 0.7)
9	2.0	1.5	20	6.17 (± 0.4)	93.7 (± 3.3)	44.2 (± 1.3)
10/βG-Spray[a]	2.0	2.5	10	10.3 (± 0.0)	93.5 (± 5.5)	39.8 (± 0.1)
11	2.2	1.0	5.0	5.50 (± 0.1)	41.2 (± 1.1)	39.6 (± 0.4)
12	2.2	1.0	15	5.20 (± 0.1)	57.2 (± 8.3)	44.2 (± 0.2)
13	2.2	2.0	5.0	8.60 (± 0.3)	75.2 (± 3.0)	39.1 (± 0.3)
14	2.2	2.0	15	8.85 (± 2.1)	97.2 (± 9.6)	43.1 (± 0.3)
15	2.4	1.5	10	5.74 (± 0.4)	89.6 (± 1.5)	42.1 (± 0.0)
βG-Gel[b]	2.0	1.8[c]	20	30.56 (± 0.6)	172.09 (± 4.2)	40.1 (± 0.2)

[a]The selected spray formulation
[b]The commercial product
[c]High MW CMC (All other formulations contained Medium MW CMC)
[d]Center point; n = 6

All formulations were confirmed to be hydrogels (Table 1), as the phase angle was below 45° [44]. The melting temperature, determined from the "oscillation temperature ramp protocol", was 39.8 °C (SD ± 0.1) and 40.1 °C (SD ± 0.2), for the finally selected βG-Spray and βG-Gel, respectively (Table 1). A βG-concentration-range between 1.6 and 2.4 % (w/w) was investigated. As expected, the lowest concentration of βG (1.6-1.8 % (w/w)) gave weaker and less versatile gels~~, as compared to the higher concentrations~~ that would be more prone to slip off the application site/wound. ~~As in the βG-Gel,~~ CMC was the selected thickening agent and glycerol was applied as a humectant in all spray formulations as in the commercially available βG-Gel. ~~But~~However, since a less viscous ~~and sprayable~~ formulation was targeted to make the spray formulation sprayable, a CMC with a lower molecular weight (MW 250,000) was used ~~in the spray formulations;~~ compared to in the βG-Gel ~~whereas a CMC with higher molecular weight~~ (MW 725,000) ~~was used in the βG-Gel (Table 2 and 3)~~. ~~Thus~~This is likely why~~,~~ a higher concentration of CMC was found to be ~~optimal~~ desirable for the βG-Spray; 2.5 % (w/w) as compared to 1.8 % (w/w) in the βG-Gel (Table 2). A~~n even higher~~ CMC concentration~~,~~ of 4.0 % (w/w)~~,~~ was applied for the NoβG-Spray (Table 2). This higher CMC concentration was selected since βG was lacking in this carrier control spray formulation, and more CMC was needed to compensate for the missing viscosity contribution from βG (Table 3). A glycerol ~~The~~ concentration range ~~investigated for glycerol was~~ from zero to 20 % (w/w) was investigated. ~~The high~~ Glycerol seemed to increase the melting point (Table 1), and considering that the glycerol concentration ~~corresponds to the content~~ in the βG-Gel is 20 % (w/w) and ~~; whereas, in~~ a previously tested spray formulation with the same active ingredient contained 10 % (w/w) glycerol ~~was applied [40].~~ [33], a similar concentration range would be preferable to compare the results. ~~The βG-Spray (f~~Formulation 10 ~~,~~ (Table 1)~~)~~ ~~also contained 10 % glycerol. With this glycerol concentration and the highest CMC concentration (2.5 %~~

~~w/w) in the test matrix (generated by Design-Expert®), and the similar βG concentrations as βG-Gel,~~ had a glycerol content of 10 % (w/w), and the same βG-concentrations to the βG-Gel. This similarity, as well as the seen favorable rheological features of Formulation 10 with a relatively high yield stress point of 93.5 Pa (SD ± 5.5), and a melting point very similar to the βG-Gel formulation, made Formulation 10 the choice for further studies as our βG-Spray candidate. ~~was obtained. Thus, the formulation 10, containing 2.5 % (w/w) CMC combined with 2.0 % (w/w) βG, and 10.0 % (w/w) glycerol was selected for the βG-Spray (~~The compositions of the selected Spray candidate, the carrier control as well as for the βG-Gel, are given in Table 2~~)~~.

Table 2. Composition of the formulations selected for further testing.

Formulation	βG (%, w/w)	CMC (%, w/w)	Glycerol (%, w/w)	H_2O (%, w/w)
βG-Gel	2.0	1.8[b]	20.0	76.2
βG-Spray	2.0	2.5[a]	10.0	85.5
NoβG-Spray	-	4.0[a]	10.0	86.0

[a]Medium MW CMC (MW 250,000)

[b]High MW CMC (MW 725,000)

The ~~two~~ selected spray formulations (Table 2) were tested for their rheological stability over a period of 56 weeks (Table 3). The NoβG-Spray formulation had a $G' < G''$ at every time point, and thus ~~was~~ classified as a viscous solution rather than a gel, with no yield point. For the same formulation, the storage modulus was lower than the loss modulus, and subsequently the phase angle was over 45°, indicating fluid behaviour [45,46]. The phase angle of the NoβG-Spray formulation was 84.4° (SD ± 1.12) at week one and did not change ($p>0.05$) at any

sampling point throughout the 56 weeks test period (Table 3). The measured decrease in phase angle for the βG-Spray formulations shows an increase in the elastic modulus, indicating a strengthening of the gel structure. This observation was supported by the increase in yield ~~point~~ stress during storage~~, from 107.4 Pa (SD ± 4.51) measured on week one, to 124.9 Pa (SD ± 2.07), 123.8 Pa (SD ± 4.87) and 126.0 Pa (SD ± 3.51) observed at week 14, 26 and 56, respectively~~ (Table 3). The increased yield ~~point~~ stress indicates ~~the~~ that more force was needed for the gel to ~~show~~ obtain a liquid behavior. Despite the observed increased gel stiffness and increased energy needed to break the gel during storage, both formulations were confirmed to be sprayable after 56 weeks, using both the 45 and the 140 µL per dose actuators. In conclusion, the NoβG-Spray and the βG-Spray, were judged stable and appropriate formulations for use in further studies as the spray dressing candidate and ~~negative~~ a carrier control, respectively.

Table 3. Stability of spray formulations tested over 56 weeks.

Week	βG-Spray		NoβG-Spray	
	Phase angle degree δ (˚)	Yield point (Pa)	Phase angle degree δ (˚)	Yield point (Pa)
1	9.41 ± 0.52	107.4 ± 4.51	84.4 ± 1.12	NA
2	8.56 ± 0.31	116.4 ±3.73	85.3 ± 0.26	NA
6	8.39 ± 0.56	116.6 ± 4.90	85.3 ± 0.03	NA
14	7.95 ± 0.18[a]	124.9 ± 2.07[a]	84.9 ± 0.41	NA
26	8.40 ± 0.35[a]	123.8 ± 4.87[a]	84.9 ± 0.37	NA
56	7.14 ± 0.15[a]	126.0 ± 3.51[a]	83.3 ± 1.73	NA

[a]$p < 0.05$ vs week 1

3.2 Fluid donation and absorption

The three formulations; βG-Spray, NoβG-Spray and βG-Gel, were found to have similar fluid donation capacities (i.e., 9.2 % ± 0.5 [SD], 6.5 % ± 1.0 [SD] and 8.7 % ± 0.4 [SD], respectively), whereas the βG-Gel formulation had more than twice the absorption capacity as compared to the spray formulations (Fig. 4).

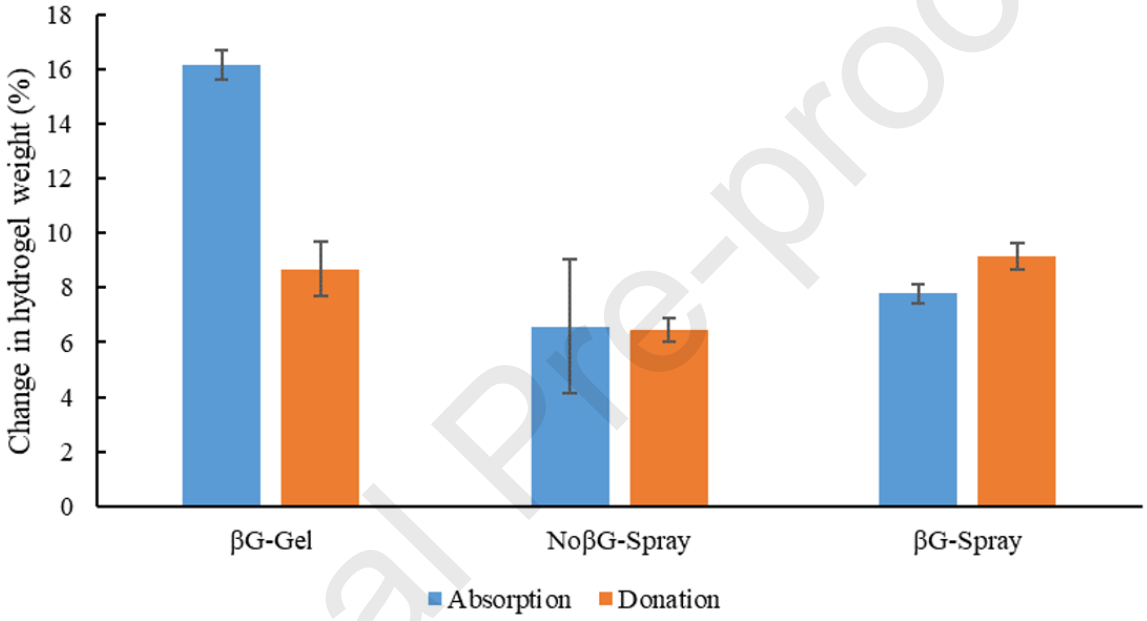

Figure 4. Fluid absorption and donation properties of the sprayable formulation (βG-Spray), the carrier control (NoβG-Spray) and the comparator dressing formulation (βG-Gel). n = 5 (% mean, ± SD).

3.3 *In vitro* cytotoxicity

The *in vitro* toxicity of the formulations was tested at t~~T~~hree different concentrations ~~were investigated~~ (1, 10, 100 µg/mL~~),~~). As shown in Fig. 5, only ~~and~~ the median concentration of the βG-Spray induced a moderate cell toxicity with a survival of approx. 86%. ~~however~~However, no toxicity was seen in any of the other formulations at any concentrations.

This also included the NoβG-Spray formulation, with the ~~a~~ higher CMC~~concentration of CMC at any of the tested concentrations~~ (~~Fig. 5~~Table 2). Thus, no dose dependent toxicity was observed for any of the formulations in the *in vitro* toxicity study.

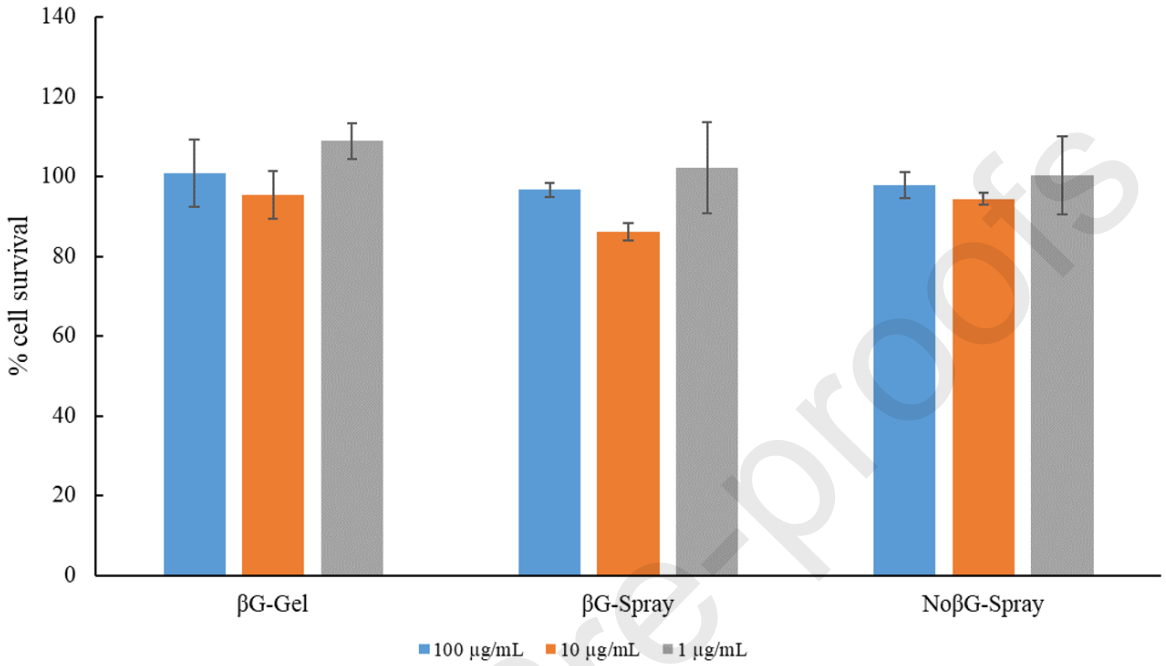

Figure 5. Cytotoxicity of the formulations to HaCaT keratinocytes assessed using the MTT-assay. Each formulation (βG-Gel, βG-Spray and NoβG-Spray) was tested at three concentrations (1, 10, and 100 µg/mL). Results are given as mean of two independent experiments (% mean, ± SD). Non-treated cells under similar condition are considered as 100% viable and not shown here.

3.4 *In vivo* wound healing

3.4.1 Macroscopic analysis

The impact of the three formulations on wound closure was investigated in full-thickness excisional wounds. These wounds were created in the dorsal flank skin of healing impaired diabetic *db/db* mice.

To assess the wound healing process, sScaled digital photographs of each wound were taken at each assessment point, and the o. Overall wound closure (% of original wound area remaining with time), as well as and the contributions of contraction and re-epithelialization were calculated from these images (Fig 6). Representative examples showing the closure of wounds in each treatment group over time are given in Appendix 1, Supplementary Fig. S2.

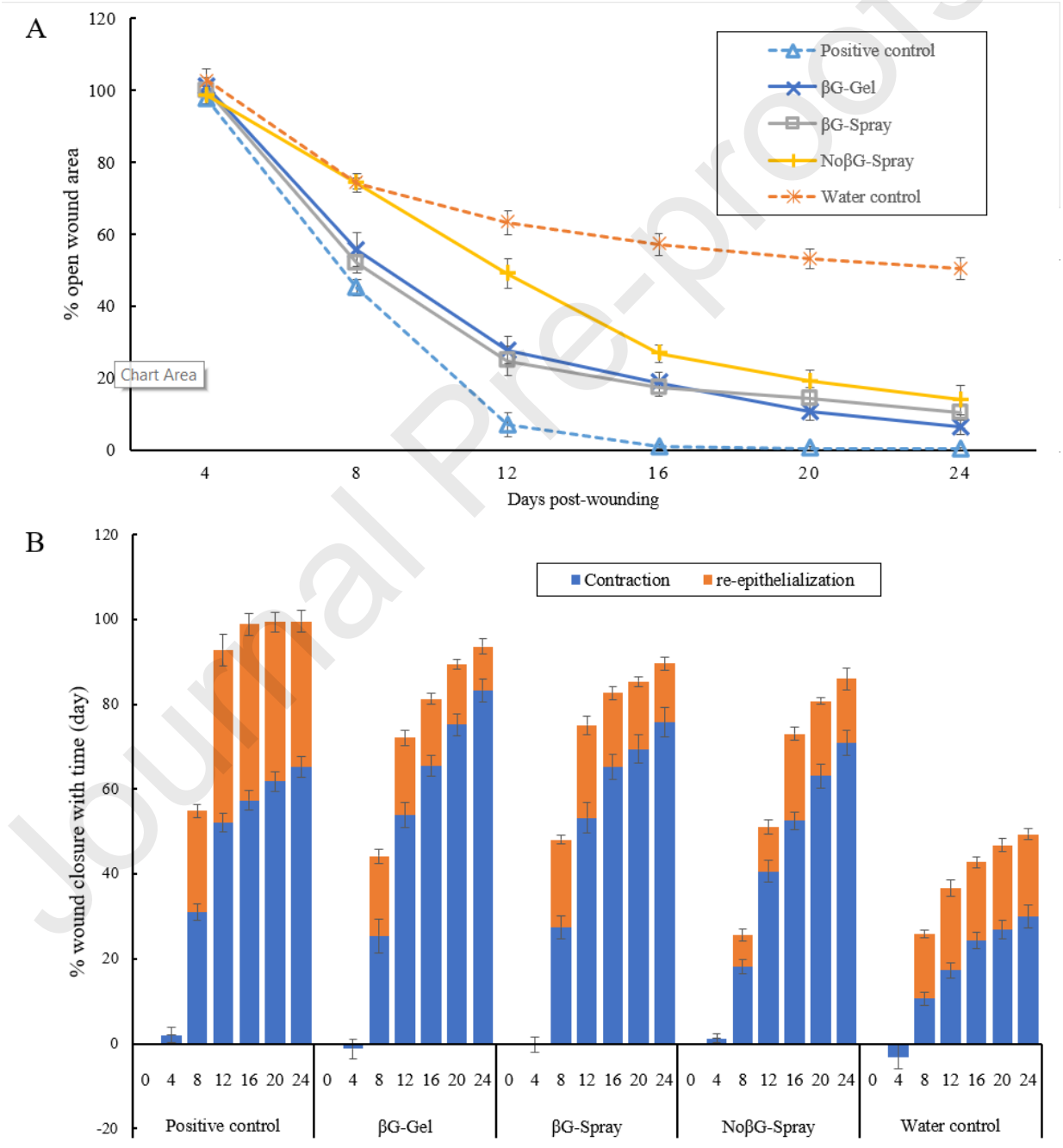

Figure 6. Impact of treatment on wound closure over the 24-day study period. A) Remaining open wound area (%) with time, B) Wound closure (%) and the relative contribution of contraction and re-epithelialization to total wound closure. Positive control (10 µg PDGF-BB and 1 µg TGF-α). (% mean, ± SEM) (n = 10).

As shown in Fig. 6, tThe closure of wounds treated with the βG-Spray was investigated over the course of the study and was investigated and compared to that of wounds infor the groups in receipt of: five different treatments; the investigated βG-spray formulation (βG-Spray), the carrier spray control spray formulation alone (NoβG-Spray); a commercial product (βG-Gel); and a positive control (PDGF-BB+TGF-α) and a negative control (water for injection) treatments (Fig. 6). During the study timeframe, aAll treatments resulted in significantly accelerated wound closure (p<0.05) during the study timeframe, when compared to the negative water treatment control (p<0.05). This was most apparent and sustained with the positive control, with significantly greater wound closure observed at all assessment points. Treatment with the βG-Spray resulted in significantly greater levels of closure ($p<0.05$) than with the carrier spray alone (NoβG-Spray) on post-wounding days 8, 12 and 16. When treatment with the βG-Spray treatment was compared to application of the commercially available βG-Gel preparation, very similar wound closure profiles were observed (Fig 6A). Treatment with the control spray (NoβG-Spray), which has no βG component, also encouraged the wound closure process when compared to negative control (water for injection) treatment.

Wound closure was also considered in terms of its components; contraction and re-epithelialization (Fig. 6B). Here, closure by contraction was found to be the main closure mechanism for all treatment groups, with improvement in re-epithelialization playing a less significant role. All treatments resulted in A significantly elevated wound contraction

(p<0.05) was observed for all treatment ~~at all assessment points~~ from day 8 onwards, ~~when~~ compared to the negative control ~~(~~Fig. 6B)~~(p<0.05)~~. Compared to positive control treatment, the βG-Gel and the βG-Spray treatment resulted in significantly greater contraction from day 16 and day 24 and onward, respectively.~~onwards, while βG-Spray treatment resulted in a significantly greater contraction on day 24, only~~. The level of contraction observed with βG-Spray and βG-Gel was indistinguishable throughout the study, whereas the βG-Spray treatment ~~was~~ gave a significantly greater contraction than ~~that following application of~~ the carrier spray ~~alone~~ (NoβG-Spray) on days 8, 12 and 16 (p ~~<~~ 0.05)~~, and indistinguishable from that following βG-Gel treatment throughout the study~~. When re-epithelialization was considered, positive control treated ~~wounds~~ displayed the most rapid and most extensive re-epithelialization of all treatment groups, with ~~— and reached~~ a peak in re-epithelialization of ~ 45 % on post-wounding day 12. Similar to the contraction levels, t~~T~~he ~~levels of~~ re-epithelialization ~~observed in response to the~~from βG-Spray and βG-Gel treatments were found to be very similar ~~to one another~~, and both ~~were~~ gave significantly greater re-epithelialization (p<0.05) compared to ~~than that in response to~~ the NoβG-Spray treatment on post-wounding days 8 and 12 ~~(p<0.05)~~.

Animals treated with βG-Spray formulation did not show any signs of adverse effects~~events~~ during the experimentation period;~~,~~ ~~and~~ wound healed in a similar fashion to that observed ~~with~~ for the commercial gel product; βG-Gel.

3.4.2 Histological analysis

Our histological investigations showed that t~~T~~he amount of granulation tissue formed within wounds ~~was found to vary~~ varied between the treatment groups (p<0.05). As shown in Fig. 7A, a~~A~~ll treatments resulted in greater granulation tissue deposition than the negative control (water for injection) ~~treatment (Fig. 7A).~~. While both βG-Gel and βG-Spray treatments resulted in greater mean granulation tissue depths compared to the carrier spray ~~alone~~ (NoβG-

Spray), no statistically significant differences were detected between these treatments. Interestingly, all treatments other than negative (water) control treatment gave rise to greater mean granulation tissue depths compared to positive control treatment. This reduced granulation tissue depth in positive control wounds is probably explained ~~in terms of~~by an increased granulation tissue maturity rather than reduced deposition – as granulation tissue compacts as it matures.

When histological re-epithelialization was considered (Fig. 7B), the greatest re-epithelialization was seen in positive control treated wounds and the lowest in wounds treated with the negative (water) control ($p<0.05$). High levels of re-epithelialization were also observed with βG-Gel and βG-Spray; but only the former was found to be significantly greater than that in response to negative control treatment ($p<0.05$). While both βG-Gel and βG-Spray were found to have re-epithelialized to a greater extent, neither proved to be significantly greater than that observed with the carrier spray (NoβG-Spray).

Collagen deposition within granulation tissue was found to be highest in the group treated with the positive control ~~wounds~~ and lowest in negative control treated wounds, in both the central wound and marginal regions. This proved to be statistically significant in the central wound region only ($p<0.05$). While both βG-Gel and βG-Spray treatments resulted in greater mean collagen deposition values than the NoβG-Spray and negative control treatments, no statistically significant differences were detected (Fig. 7C).

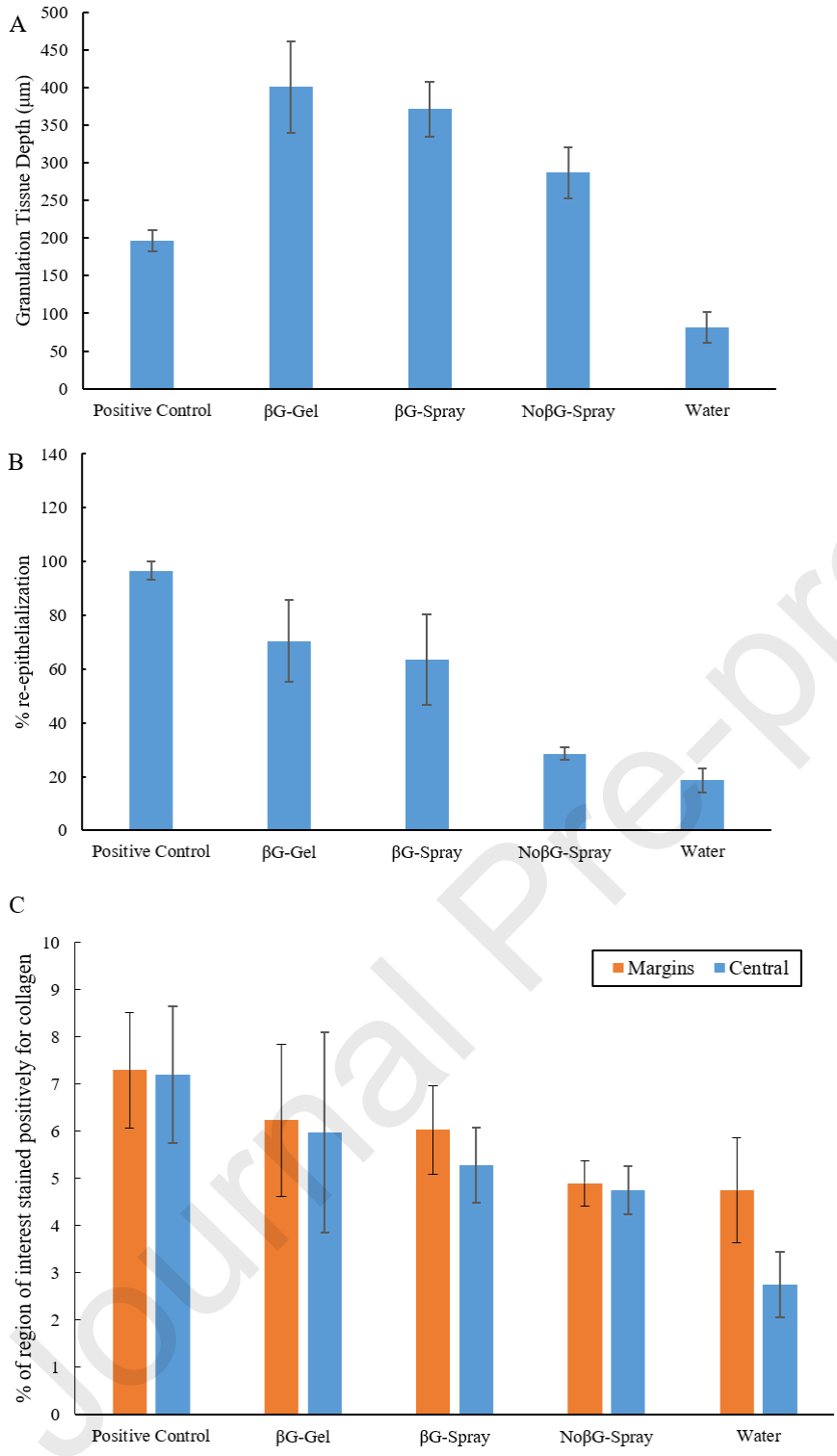

Figure 7. Impact of treatment on post-wounding day 24, with regards to A) granulation tissue depth, B) 'histological' re-epithelialization and C) collagen deposition in granulation tissue both in margins and central wound. Positive control (10 µg PDGF-BB and 1 µg TGF-α). (mean, ± SEM) (n = 4).

4. Discussion

Hydrogels are recommended for use in the treatment of chronic wounds, as they are able to cleanse wounds by rehydrating dead tissues and assist in autolytic debridement [5]. Furthermore, hydrogels can reduce perceived pain and promote re-epithelialization by providing a moist wound healing environment [34]. Clinical studies have suggested that βG-hydrogels promote the healing of chronic wounds by two mechanisms; by i) the before mentioned favorable environmental effects of the hydrogel in the wound, and ii) the βG components activation of macrophages – which are known to orchestrate the wound healing process [26]. As far as we know, spray-application of βG-hydrogels represents a novel treatment of chronic wounds. The ease by which spray-formulations can be applied makes the spray format suitable for administration by both medical professionals and patients themselves, and particularly interesting for treatment of large or hard to access wounds [30,47]. Thus, a spray formulation will offer advantages in the treatment of certain wounds as compared to the currently commercially available βG-Gel, a semisolid hydrogel usually spread on the wound surface with a gloved finger.

~~In order f~~For a hydrogel to be both sprayable and retained at the wound surface, it must possess certain rheological characteristics. Thus, ~~the first step of these investigations was to develop a formulation with these necessary characteristics. For this, a~~a multi-factorial design matrix was applied for the screening study (Table 1), with preselected concentrations ranges for the three ingredients. The ingredients included in the spray formulations; βG, ~~carboxymethyl cellulose (~~CMC~~)~~ and glycerol, were selected based on the composition of a marketed βG hydrogel product (Woulgan® Gel) – referred to as βG-~~gel~~ Gel in this article. The active ingredient, βG, is a water-soluble β-1,3/1,6-glucan isolated from baker's yeast (Saccharomyces cerevisiae~~), ~~). βG was provided in the form of ~~as~~ a sterile hydrogel,

containingwith a final concentration of 2.5 % (w/w) βG in water [38,48]. Thus, a higher βG-concentration than 2.5 % (w/w) is not achievable for the final spray formulations. This water soluble βG has an weight-average MW of about 7 x 10^5 g/mol(in an aqueous solution), with a wide size distribution, and forms a tertiary triple-helix structure in an aqueous solution [49]. This higher order of structure is thought to be vital to elicit immunological activity, but the binding of β-glucans to the immune receptors is still not fully understood [50]. In this work, we chose to evaluate βG at concentrations ranging from 1.6 to 2.4 % (w/w) was evaluated. In a previous study, 1.0 % (w/w) a βG was assessedjudged to be the lowest concentration necessary for optimal wound healing efficacy [33]; and 2.0 % (w/w), the βG-concentration in the βG-Gel, has been shown to be effective in the clinical setting [23,26].

The commercial βG-Gel contains a high viscosity 725,000 k-Dalton CMC as a thickening agent; but, as we aimed to develop a less viscous, sprayable product, a CMC with a MW of 250 k,000 Dalton was selected for the spray formulations. CMC is a highly water-soluble anionic polysaccharide of ether cellulose, with a long tradition as of useingredient in topical pharmaceutical formulations [5,51,52]. The swelling and mucoadhesive properties of the polymerCMC make it an excellent ingredient for wound dressings. After some preliminary experimentation (results not shown), a CMC concentration ranges between 0.5 and 2.5 % (w/w) were investigated in this study. The third ingredient, glycerol**,** was added as a humectant in a concentration range from zero to 20 % (w/w). This range was chosen since the commercially marketed βG-Gel contains 20 % (w/w) glycerol. All the 15 spray formulations were proven to be sprayable with the selected container and pump system (the nozzle Comfort® spray system). Resistance to friction between the wound and the secondary dressing desire a relatively high yield stress [15]. As seen in Table 1, a yield stress point of 93.5 Pa (SD ± 5.5) was obtained for Formulation 10, corresponding to the finally selected βG-Spray. with 10 % (w/w) glycerol, 2.5 % (w/w) CMC and 2.0 % (w/w) βG. This higher yield stress is

thought to be due to the inclusion of the high(est) CMC-concentration of 2.5 % (w/w) in this formulation. However, all ingredients seem to increase the yield stress, and βG even more than CMC~~,~~. Although~~has~~ ~~spray f~~Formulation 14 (Table 1), with 15 % (w/w) glycerol, 2.0 % (w/w) CMC and 2.2 % (w/w) βG, was the spray formulation with ~~had~~ the overall highest yield stress of 97.2 (± 9.6)~~among the spray formulations, of 97.2 (± 9.6). But, since this difference~~ Formulations 10 with a yield stress of 93.5 Pa (SD ± 5.5) was preferred formulation for further testing, because i) this difference in yield stress was not statistically ~~different~~significant ~~between Formulations 10 and 14~~, and ii) Formulations 10 had the same concentrations of βG as the as the commercial product, βG-Gel.~~, Formulations 10 was the preferred formulation.~~ The melting temperature of the formulations was determined using the oscillation temperature ramp protocol (Table 1). All formulations had a melting temperature higher than normal skin temperature (33 °C). ~~Since the f~~Formulation that lacked glycerol (Formulation 7) had the lowest melting temperature recorded (34.6 °C), and ~~also since all~~ formulations containing only 5% glycerol had a melting point ≤ 39.6 °C (Table 1).~~,~~ Thus, ~~it seems like~~ glycerol increased the melting temperature of these hydrogels. This is in accordance with other publications where ~~can be explained by~~ glycerol has shown to stabilis~~e~~ing the polymer gel network through the formation of hydrogen bonds [53]. The βG-Spray and the βG-Gel were found to have a melting point of 39.8 °C (SD ± 0.1) and 40.1 °C (SD ± 0.2), respectively. This indicates that neither formulation would melt when applied to the wounds.

As shown in Table 2, 4.0 % (w/w) CMC was selected for the NoβG-Spray, the carrier control ~~formulation to be~~ applied ~~as a reference formulation~~ in the *in vivo* studies. Although the total polymer concentration (w/w) is similar to that of the βG-Spray formulation, the NoβG-Spray formulation ~~was found to be much more liquid~~had a lover viscosity than βG-Spray (Table 3).

Thus, the active ingredient, βG, is ~~obviously~~ forming a more rigid polymer network than CMC with the applied (MW =250,000). This means that the carrier control, NoβG-Spray, did not only lack the active ingredient, βG, but also ~~displays~~ displayed less desirable rheological features as a wound healing product.

A long shelf life, preferably at room temperature, is always aimed for when developing new medical products. In this study, the stability was assessed for 56 weeks, by recording the rheological propertied of the two selected spray formulations (the βG-Spray and NoβG-Spray) ~~for more than one year (56 weeks)~~. Rheological changes of the product will not only reflect chemical and physical degradation of the product, but ~~One good reason for using rheological measurements to assess stability, is that a change in rheological behaviour~~ might also change the dressings ability to be retained at the wound surface, which is critical for the dressing to assert its effect [15]. As shown in Table 3, ~~We observed~~ a delayed onset of the 3D gel-network formation by the βG polymers was observed from the yield point assessments ~~(Table 3)~~. ~~This corresponds well with previously tested βG-Spray formulations containing Carbopol as a thickening agent [39]. However,~~ . Tthe ~~measured~~ phase angles of the βG-Spray formulation did not ~~show any~~change significantly ~~difference~~ between week 1 and week 2 ($p>0.05$). ~~(Ta~~ble 3). ~~Thus, week 1 was defined as the starting point for the stability study for both formulations.~~ The significant reduction in phase angle and increase in yield point after 14 weeks of storage suggests strengthening of the βG-polymer network. Since the sprayability might be affected by ~~the increased~~ gel stiffness, the sprayability was ~~we~~ retested and confirmed ~~that~~ for both spray-formulations ~~were sprayable also~~ after 56 weeks of storage. Thus, we concluded that both the βG-Spray and NoβG-Spray formulations were stable over 56 weeks, in terms of both sprayability and gel strength. Further characterisation and testing

were therefore encouraged for both formulations, despite the liquid behavior of the NoβG-Spray.

A moist wound environment is considered to be the best environment for wound healing to occur [5]. Maintaining a favorable moist balanced ~~moist~~, and avoiding a too~~rather than~~ wet or dry environment, is essential to promote wound debridement and provide a matrix for skin regeneration [15]. Hydrogel dressings should therefore be able to donate moisture to dry wounds and absorb excess moisture under exudative conditions. Since dry to low exuding wounds were the target wound type for the spray format, t~~T~~he similar fluid donation capacity and the lower fluid absorption capacity of the βG-Spray formulations as compared to the βG-Gel (Fig. 4), was considered a positive outcome~~, since dry to low exuding wounds are the target wound type for spray formulations~~. The higher absorption capacity of the βG-Gel formulation, as compared to the βG-Spray formulation, supports its use in more highly exuding wounds. This is in accordance with recommended use of this commercial βG-Gel, which is indicated for low to moderately exuding chronic wounds [26]. All three formulations in this study showed good buffering capacity for moisture handling in the wound bed, with a capacity to both absorb and donate more than 6 % (w/w) liquid/wound exudate. The ability to donate fluid to wounds helps with autolytic debridement; whereas~~,~~ the ability to absorb wound exudate and debride slough helps healthy tissue to re-epithelialize [14]. Providing too much moisture to wounds can lead to maceration of peri-wound tissue which can extend healing time [14]. The importance of selecting a suitable thickening agent to formulate a hydrogel for wound healing applications, was highlighted by previous work performed by our group [33]. In this study, Carbopol (Lubrizol, USA) was selected as the thickening agent. Fluid affinity investigations of these Carbopol formulations showed a low absorptive capacity (0.5 % (w/w)) combined with a high fluid donation capability (17 % (w/w)). As a

consequence, excessive hydration, tissue maceration and impaired wound closure ~~was~~ were observed. ~~In this current study, like with the Carbopol study [40], t~~The βG-Gel formulation and the βG-Spray formulations differ with regards to glycerol concentrations, ~~which was~~with 20 % (w/w) and 10 % (w/w), respectively. This might contribute to the higher absorption of fluid by the βG-Gel, as glycerol have been reported to absorb three times its own weight in water [54]. Although being a humectant, glycerol is also hygroscopic and a viscous liquid that acts as a wetting agent when swelling the polymers into a hydrogel network. The most likely explanation for the difference in absorption ability between these two formulations (Fig. 4), is therefore the different CMCs applied. It appears that the high molecular mass CMC applied in the βG-Gel formulation forms a hydrogel structure with a higher absorption capacity than the medium molecular mass CMC applied in the sprayable formulations. The two spray formulations investigated, βG-Spray and NoβG-Spray, showed a very similar fluid affinity profile. Thus, the polymer network and interaction of βG and CMC seem to have similar absorption and donation features as CMC alone when the medium molecular mass CMC is used. The dry polymer mass in the βG-Spray was 4.5 % (w/w)~~; 2.5 % CMC and 2.0 % βG~~, whereas the NoβG-Spray formulation containing 4.0 % CMC (Table 2). Increasing the polymer concentration of the NoβG-Spray formulation, may therefore ~~to 4.5 % for both may~~ have made the fluid affinity profiles even more similar. However, these results show that all formulations have the desired ability to both donate and absorb fluid.

The biocompatibility of a medical product is essential to ensure its safe use in the clinical setting. Keratinocytes play a crucial role in epidermal tissue regeneration, and HaCaT cells (a spontaneously immortalized, human keratinocyte cell line) therefore provide a useful *in vitro* tool for determining potential cellular toxicity. Toxicity assessments based on the survival of HaCaT cells, usually claim substances are ‘non- cytotoxic’ with greater than 90 % cell

survival, 'moderately cytotoxic' with 80 to 90 % cell survival, and 'significant cytotoxic' with less than 80 % survival [55]. The MTT assay findings generated in this work (Fig. 5) showed that none of the formulations tested (βG-Gel, βG-Spray or NoβG-spray) displayed any significant cytotoxicity at the highest concentration tested. The lack of cytotoxicity to HaCaT cells, with NoβG-Spray at any of the tested concentrations (Fig. 5), correlates well with other studies that have investigated CMC and βG [56,57]. These positive biocompatibility findings were confirmed by the apparent lack of cellular toxicity or other adverse effects in subsequent diabetic mouse wound healing studies.

The diabetic *db/db* mouse delayed wound healing model, ~~as~~ also previously described by our group [27,33], is a useful tool ~~has a long history of use~~ in the pre-clinical evaluation of wound healing therapies [58]. Using this model, βG-Spray was found to promote wound healing at both the macroscopic and histological levels, to a level largely similar to that of the commercial comparator βG-Gel. At the macroscopic level, both βG formulations were found to give rise to significant improvements in overall wound closure and its components contraction and re-epithelialization, when compared to the ~~vehicle/~~carrier control formulation NoβG-Spray.

The ~~observed~~ wound healing rates in both the βG-Spray and the βG-Gel treatment groups were observed to decrease around day 12 post-wounding (Fig. 6A). As these formulations were only administered on day 0, 2, 4, 6 and 8 post wounding, it is possible that the healing rate could have been maintained, if these treatments had continued to be applied more times through ~~at a later time points in~~ the study. In a study by Berdal and co-workers, the authors reported a dose frequency dependency of the β-glucan used on wound closure rate, favouring a more frequent administration of β-glucan for increased wound closure [59]. Consequently,

the impact of longer-term ~~dosing~~ treatments should be investigated closer ~~with a view~~in future ~~to~~ optimisation of~~zing the beneficial effects of~~ these formulations.

Interestingly, the vehicle formulation (NoβG-Spray) ~~was~~ also ~~found to give~~gave rise to significant improvements in the overall wound closure, and promoted both wound contraction and granulation tissue formation relative to the negative control treatment ~~used in this study~~ (~~i.e.,~~ water for injection). This may be explained ~~in terms of~~by the fluid handling properties of CMC, and/or possibly also by ~~a direct effect of~~ CMC's overall favorable effect on the wound healing process [36,51]. CMC is used in numerous commercially available wound treatments and has also previously been shown to increase the rate of wound healing compared to control treatment. Fluid handling of the applied formulation seems to be very important, taking into consideration the poor *in vitro* performance of the previously tested Carbopol-based spray formulations investigated ~~previously~~ [33], as well as the liquid behaviour of the NoβG-Spray formulation (Table 3). ~~With regards to the Carbopol-based spray formulations, these formulations contained the same active ingredient and was tested using the same *in vivo* wound healing model. But, this present CMC-containing βG-Spray formulation had a more than 10 fold higher fluid absorption capacity as compared to the Carbopol-based formulation.~~ It is thus suggested that the observed adverse effects of the Carbopol-containing formulation noted in our previous work [33], was a consequence of an inadequate fluid absorption and extensive fluid donation. If ~~so~~this is the case, the choice of thickening agent ~~selected for the formulation~~ seem to be of paramount importance for its clinical success in wound healing. The NoβG-Spray, although lacking the active ingredient, enabled formation of a viscous solution, which can exhibit beneficial physical protection, and an adhesion-free cover of the sensitive wound tissue, acting on improved wound healing.

When the impact of treatment was considered at the histological level (Fig. 7), both βG formulations gave rise to improvement in wound healing, though largely non-significant relative to NoβG-Spray. All the hydrogel formulations were found to encourage granulation tissue formation, re-epithelization and collagen deposition in the central wound relative to the water-control ($p<0.05$). As noted previously, all three hydrogel formulations (βG-Gel, βG-Spray and NoβG-Spray) acted to promote wound closure primarily by promoting wound contraction rather than re-epithelialisation, and observation that parallels previous βG work by our group [27,33]. As contraction is driven by the compaction of granulation tissue this may suggest that these formulations act to encourage wound closure by promoting the formation, and/or quality, of granulation tissue. Granulation tissue formation is known to be orchestrated by macrophages, and β-glucans are immunological response modifiers, that can activate wound macrophages, which may explain the beneficial effects of β-glucans noted in this study, in our previous work, and by others [23,28,37,60–63]. Since the histology was obtained at the final day of the *in vivo* study, a very pronounced observed effect from β-glucan could not be anticipated, taking into account the macroscopic analysis at the same time point (Fig. 6). Thus, the histology findings fully support the reported microscopic analysis in this *in vivo* wound healing study.

The data generated from this *in vivo* study clearly demonstrates the beneficial effects of a sprayable βG-supplemented hydrogel on the mammalian wound healing process, and highlights its significant potential as a treatment for chronic wounds.

5. Conclusion

A sprayable hydrogel formulation comprising the immunomodulatory βG, soluble β-1,3/1,6-glucan, isolated from baker's yeast (*Saccharomyces cerevisiae*), was successfully prepared.

The new spray formulation showed equivalent wound closure time as the commercially available semisolid hydrogel formulations. Since the spray is designed for multiple applications particularly targeting bigger and dryer wounds than the current available semisolid βG-formulations, this new βG-Spray will expand βG's clinical use, as the spray format will be beneficial for different patient groups and wounds. ~~Moreover, this unique active ingredient would expand its clinical relevance.~~

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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TABLE- AND FIGURE LEGENDS

Table 1. Rheological characteristics of the β-glucan containing formulations. All measurements are an average of two independent experiments except the center points (± SD).

Table 2. Composition of the formulations selected for further testing.

Table 3. Stability of spray formulations tested over 56 weeks.

Figure 1. Illustration of the wound healing parameters and terminology used to assess the progress of wound closure during the study. A) A wound on day 0 (day of surgery), B) The same wound on post-wounding day 12.

Figure 2. Post-wounding day 24 diabetic mouse wound section stained with haematoxylin and eosin (H&E) showing: "re-epithelialization from the wound margins" (A & B), a "central non-epithelialized region" (C), and "granulation tissue depth" (d) at ~~9~~ nine points across the wound.

Figure 3. A) Diabetic mouse wound section (day 24) stained specifically for collagen using with Picrosirius Red, with 3 (1000 ~~μm~~ x 1000 μm) sub-regions shown (bar 500 μm); B) enlarged right-marginal region - dark red staining is mature collagen (bar 100 μm); C) Image B manipulated to display collagen staining only (bar 100 μm).

Figure 4. Fluid absorption and donation properties of the sprayable formulation (βG-Spray), the carrier control (NoβG-Spray) and the comparator dressing formulation (βG-Gel). n = 5 (% mean, ± SD).

Figure 5. Cytotoxicity of the formulations to HaCaT keratinocytes assessed using the MTT-assay. Each formulation (βG-Gel, βG-Spray and NoβG-Spray) was tested at three concentrations (1, 10, and 100 µg/mL). Results are given as mean of two independent experiments (% mean, ± SD). Non-treated cells under similar condition are considered as 100% viable and not shown here.

Figure 6. Impact of treatment on wound closure over the 24-day study period. A) Remaining open wound area (%) with time, B) Wound closure (%) and the relative contribution of contraction and re-epithelialization to total wound closure. Positive control (10 µg PDGF-BB and 1 µg TGF-α). (% mean, ± SEM) (n = 10).

Figure 7. Impact of treatment on post-wounding day 24, with regards to A) granulation tissue depth, B) 'histological' re-epithelialization and C) collagen deposition in granulation tissue both in margins and central wound. Positive control (10 µg PDGF-BB and 1 µg TGF-α). (mean, ± SEM) (n = 4).