Biodegradable soy wound dressings with controlled release of antibiotics: Results from a guinea pig burn model

Dana Egozi a, b, Maya Baranes-Zeevi c, Yehuda Ullmann b, d, Amos Gilhar b, Aviad Keren b, d, Elias Matanes d, Israela Berdichevsky b, Norberto Krivoy b, Meital Zilberman c, *

a Department of Plastic Surgery, Kaplan Medical Center, Rehovot, Israel
b Faculty of Medicine, Technion – Israel Institute of Technology, Haifa 32000, Israel
c Department of Biomedical Engineering, Tel-Aviv University, Tel-Aviv 69978, Israel
d Department of Plastic Surgery and the Burn Unit, Rambam Health Care Campus, Haifa, Israel

A R T I C L E   I N F O

Article history:
Accepted 27 March 2015

Keywords:
Gentamicin
Infection
Histology
Soy protein
Wound healing

A B S T R A C T

There is growing interest in the development of biodegradable materials from renewable biopolymers, such as soy protein, for biomedical applications. Soy protein is a major fraction of natural soybean and has the advantages of being economically competitive, biodegradable and biocompatible. It presents good water resistance as well as storage stability. In the current study, homogeneous antibiotic-loaded soy protein films were cast from aqueous solutions. The antibiotic drug gentamicin was incorporated into the films in order to inhibit bacterial growth, and thus prevent or combat infection, upon its controlled release to the surrounding tissue. The current in vivo study of the dressing material in contaminated deep second-degree burn wounds in guinea pigs (n = 20) demonstrated its ability to accelerate epithelialization with 71% epithelial coverage compared to an unloaded format of the soy material (62%) and a significant improved epithelial coverage as compared to the conventional dressing material (55%). Our new platform of antibiotic-eluting wound dressings is advantageous over currently used popular dressing materials that provide controlled release of silver ions, due to its gentamicin release profile, which is safer. Another advantage of our novel concept is that it is based on a biodegradable natural polymer and therefore does not require bandage changes and offers a potentially valuable and economic approach for treating burn-related infections.

© 2015 Elsevier Ltd and ISBI. All rights reserved.
1. Introduction

1.1. Soy protein

There is growing interest in the development of biodegradable materials from renewable biopolymers, such as soy protein, for biomedical applications [1–7]. Soy protein is a major fraction of natural soybean and can be found in several soybean derivatives in different quantities, depending on the extraction method: defatted soy flour contains 50–59% protein, soy protein concentrate contains 65–72% protein and soy isolate contains more than 90% protein [8]. Soy protein has been explored mainly in the polymer, food and agriculture fields. Use of soy protein as a food source is still increasing, due to its functional and nutritional value, availability and low price [9]. In the materials industry, soy protein was studied as an adhesive and as a “green” plastic [1]. Soy protein is an abundant plant protein and has the advantages of being economically competitive, biodegradable and biocompatible. It presents good water resistance as well as storage stability [4]. Soy protein is also very versatile and its properties can be tailored by physical, chemical, or enzymatic treatments [10], such that it can provide diverse requirements for different biomedical applications. It is known that local or systemic use of soybean or its components has an Ig E induced immunogenic effect more in children and less in adults, but in spite of it the soybean is used for the preparation of an intravenous medication widely used in surgery. As always, the public should be informed in the package insert. The statistics says that soybean affects approximately 0.4% of the children’s population. Also, approximately 50% of children with soy allergy outgrew their allergy by age of 7 years. The soy protein extract used in the current study was provided by a company which sells it for the food industry and therefore it is considered as safe.

The suitability of soy protein for biomedical applications has been investigated, due to its low cost and bioactive properties [11]. Soy thermoplastics were found to be non-cytotoxic, and even encouraged cell proliferation during in vitro tests [12]. Proposed applications range from bone cement to hydrogel and membranes for wound-dressing applications. The most promising recent applications include drug delivery carrier films, temporary replacement implants and scaffolds for tissue engineering [13].

Snyders et al. [14] and Shingel et al. [15] investigated hybrid hydrogels made of poly(ethylene glycol) and soy protein for moist wound-dressing applications and concluded that these hydrogels can be considered as a safe, biocompatible and inflammatory inert wound-dressing material. Santos et al. [16] developed chitosan/soy (cht/soy)-based membranes as wound-dressing materials and showed that these new membranes possess the desired features for healing/repair stimulation, ease of handling, and final esthetic appearance, which are valuable properties for wound dressings.

1.2. Wound dressings

Various types of wounds result in tissue damage. These include burns, pressure ulcers, diabetic ulcers and trauma. The main goal in wound management is to achieve rapid healing with good functional and esthetic results. An ideal wound dressing can restore the milieu required for the healing process, while simultaneously protecting the wound bed against bacteria and environmental threats. The dressing should also be easy to apply and remove. Most modern dressings are designed to maintain a moist healing environment and to accelerate healing by preventing cellular dehydration and promoting collagen synthesis and angiogenesis [17]. However, over-restriction of water evaporation from the wound should be avoided, since accumulation of fluid under the dressing may cause maceration and facilitate infection. The physical and chemical properties of the dressing should therefore be adapted to the type of wound as well as to the degree of wound exudation.

Bacterial contamination of a wound seriously threatens its healing. In burns, infection is the major complication after the initial period of shock, and it is estimated that about 75% of the mortalities following burns are related to infections rather than to osmotic shock and hypovolemia [18]. This has encouraged the development of improved wound dressings that provide an antimicrobial effect by eluting germicidal compounds such as iodine (Iodosorb®, Smith & Nephew), chlorohexidine (Biopatch®, J&J), or, most frequently, silver ions (e.g., Acticoat® by Smith & Nephew, Actisorb® by J&J and Aquacel® by Convatec). Such dressings are designed to provide controlled release of the active agent through a slow but sustained release mechanism which helps avoid toxicity yet ensures delivery of a therapeutic dose into the wound.

1.3. About the current study

It can be said that there is an increasing need to develop new biodegradable materials for use in wound-healing applications. Soy protein is a very promising natural biodegradable material, due to the abovementioned advantages. We therefore chose to develop and study soy protein films as a platform for wound-dressing applications, in all relevant aspects. We believe that a high quality soy protein film which is hydrophilic but also cross-linked and therefore exhibits desired relevant properties may provide properties that are usually achieved using the bilayer concept.

Homogenous antibiotic-loaded yellowish films were cast from aqueous solutions. A detailed description of the preparation process was published elsewhere [19]. The antibiotic drug gentamicin was incorporated into the films in order to inhibit bacterial infection upon its controlled release to the surrounding tissue. The mechanical and physical properties of these films, the antibiotic release profiles and their antimicrobial effects as well as cellular response results, were recently published by us [19,20]. Since these films are crosslinked and also contain plasticizer (glycerol), they are strong and flexible and can be easily handled. The new gentamicin-eluting soy protein wound dressing which exhibited the best properties in the above study was selected for the current in vivo study and compared to a neat soy protein wound dressing without the drug and to a standard Melolin dressing.

The guinea pig is often used as a dermatological and infection model [21–24]. Research on guinea pigs has included
topical antibiotic treatment [25], delivery of delayed-release antibiotics [26], and investigation of wound-dressing materials [27,28]. A deep partial skin thickness burn is an excellent wound model for the evaluation of wound healing, not only for contraction and epithelialization from the peripheral area such as in third-degree burns, but also for evaluation of the recovery of skin appendages that serve as the main source for the re-epithelialization which completes the healing process. The metabolic response to severe burn in guinea pigs is very similar to the human post-burn metabolic response [29]. Furthermore, bacterial colonization and changes within the complement component of the immune system in human burn victims is analogous to guinea pigs affected by severe burns [23]. Such a model was therefore used in the current study to evaluate the effectiveness of our novel composite antibiotic-eluting wound dressing.

2. Materials and methods

2.1. Materials

Non-genetically modified soy protein (Solpro 910™, minimum 90% (w/w) protein, on a dry weight basis) was obtained as a donation from Solbar™ (Ashdod, Israel).

Glycerol (G-7893) was used as plasticizer, glyoxal (50650) was used as the cross-linking agent and the antibacterial drug gentamicin sulfate (G-1264) (450–477 g/mol, T_m = 218–237 °C) was used as the incorporated active agent (all purchased from Sigma–Aldrich, Rehovot, Israel).

Reagent kit (8-1P31-25) and calibration kit (8-1P31-01) were purchased from Sigma–Aldrich, Rehovot, Israel for analysis of gentamicin concentrations.

2.2. Preparation of the soy protein films

Films of soy protein were prepared using the solvent casting method. Soy protein solution was prepared by dissolving soy protein powder in preheated double distilled water at 55 °C and thoroughly stirred. Plasticizer (glycerol) and cross-linker (glyoxal) were then added. For the gentamicin-loaded films, gentamicin was first dissolved in 10 mL of the preheated double distilled water and added to the soy protein solution (following the addition of the cross-linker). All film parameters are presented in Table 1.

The solution was constantly stirred with a magnetic stirrer and kept at 55 °C for 30 min to allow the removal of bubbles. The solution was then stirred at room temperature for an additional 30 min to enable further removal of bubbles and initial cross-linking. Finally, the solution was cast into two low-density polyethylene plates (8.74 cm diameter) and left to dry under ambient conditions for 72 h. In order to maintain uniform thickness of the films, exactly 45 mL of solution were cast into each plate. This amount yielded films with a thickness of approximately 0.5 mm.

When dried, the films were carefully filed off the dishes and cut into 45 mm discs. Each disk was then positioned between two glass Petri dishes and subjected to thermal treatment in an oven for 24 h at 80 °C. The specimens were kept in a desiccator (RT, 30% RH) at room temperature and constant humidity until use. All specimens were sterilized with ethylene oxide and sealed in an airtight plastic bag.

2.3. In vitro gentamicin release

The soy protein films were cut into round discs (1 cm diameter), which were individually placed in glass tubes in triplicates and 3 mL of the release medium were added to each tube. The tubes with the immersed discs were sealed with plastic cups and kept in a microbiological incubator (Heraeus, B12) at 37 °C and 100% humidity under static conditions. The entire release medium from each tube was removed at predetermined time points and was replaced by fresh medium (3 mL). The removed medium was kept refrigerated at 4 °C until analyzed.

Immunoassay analysis: Gentamicin concentrations were determined using a high-speed immunoassay with an ARCHITECT i2000SR (Abbott) system. Samples were diluted with PBS (pH 7) with 0.02% sodium azide, as necessary.

2.4. In vivo burn model: procedure and treatment groups

Twenty female guinea pigs (Harlan, NL) were allocated for the study after authorization by the Technion Animal Care and Use Committee (authorization IL-092-09-2012). The animals (average weight 300 g) were raised in a pathogen-free animal facility. Following five acclimatization days, the animals were randomly divided into three groups. All animals were anesthetized by intramuscular injection of ketamine (40–50 mg/kg) and xylazine (4–5 mg/kg). After shaving the animals’ skin, a depilatory cream (Orna 19, Alpha Cosmetica Israel) was applied to complete hair removal. Two standardized deep second-degree burns were inflicted on the back of each animal on either side of the spine according to a validated method described by Kaufman et al. (1990). Iron templates (circle, D = 40 mm) were immersed in water preheated to 75 °C and then placed in perfect contact with the animal’s skin for exactly 5 s by applying light pressure. The burn area was traced onto a transparent paper as a reference for later follow-up. After infliction of the burns, each animal was seeded with 0.5 mL broth containing 1 × 10^6 CFU/mL Pseudomonas aeruginosa using a micropipette. Each group was then treated with the relevant treatment option, as follows:

Group 1 (6 animals, 12 wounds) was treated with a neutral non-adherent dressing material (Melolin®, Smith & Nephew). Melolin® consists of three layers: a low adherent perforated film, a highly absorbent cotton/acyllic pad and a hydrophobic backing layer. According to the manufacturer, it enables rapid drainage of wound exudate, thus reducing trauma to the healing tissue. The dressing was placed directly on the burn,
and was secured by an elastic adhesive bandage (Tensoplast™, Smith & Nephew).

Group 2 (7 animals, 14 wounds) was treated with our soy dressing, without antibiotics. A round disk slightly larger than the burn area (D = 45 mm) was placed directly on the burn, covered with Melolin®, and secured as described above. This dressing material was tested in order to evaluate the effect of the dressing’s texture, materials, and degradation on the wound-healing process.

Group 3 (7 animals, 14 wounds) was treated with the soy dressing which released gentamicin. The dressing materials were placed and secured as described above. These dressing materials were tested in order to evaluate the effect of antibiotic release kinetics on the wound-healing process.

Each of the animals was placed in an individual cage with food *ad libitum* and allowed to recover. The animals received analgesic treatment prior to the procedure and for 5 consecutive days (buprenorphine, 5 mg/kg per os).

2.5. Wound closure

Twelve days following the burn, wound areas were traced on a transparent paper and scale bar photographs were taken. The burn wounds were evaluated macroscopically by two quantitative parameters: (i) percentage of the original area subjected to burn which was still an open wound, and (ii) wound contraction as depicted by the total wound area (epithelialized and non-epithelialized) as a percentage of the original area subjected to burn. The measurements of the two burns within one animal were averaged to produce a single data, from which the total group average was calculated.

2.5.1. Post mortem examination

The animals were sacrificed and 1 cm² biopsies were taken from the center of the wound and immediately fixed in phosphate buffered formalin.

2.5.2. Biopsy evaluations

Following the fixation stage, biopsies were embedded in paraffin using a standard embedding protocol, and 5 µm sections were prepared using a Leica microtome. Sections were stained with hematoxylin and eosin (H&E), observed and photographed under ×20 and ×100 magnification using an Olympus upright light microscope. Wound-healing analysis was conducted in a blinded manner by two separate evaluators using a semi-quantitative grading system. The data obtained from the observers was analyzed and based on the average of the two burns within one animal to produce a single observation per animal. The sections were evaluated based on structure and content. The wound-healing criteria included epithelial coverage, epidermal thickness, epidermis-dermis attachment, neangiogenesis, number of follicles (adnexa), dermis thickness, white blood cells (WBC) and collagen. Grading was between 0 and 3: 0 absence, 1 minimal presence, 2 moderate presence and 3 extensive presence, except for WBC, where 0-extensive presence and 3-absence.

2.6. Statistical analysis

Means and standard deviation of sample (SD) were calculated for each data set. Differences between means were analyzed for statistical significance using a one-way ANOVA with the Tukey-Kramer multiple comparisons posttest (SPSS version 17.0). *p* values ≤ 0.05 were considered significant.

3. Results

3.1. In vitro gentamicin release profile

Cumulative release of gentamicin from the soy protein wound dressings presented an initial burst release (6 h) of approximately 54% followed by two phases of release. The first phase exhibited a decreasing release rate for a period of 12 days, followed by a second phase of a constant slow release rate of 0.15% drug/day 

3.2. Wound closure

Second-degree burns in guinea pigs were used as a wound-healing model for testing our novel soy wound dressing, which is a membrane that covers the wound and allows slow release of gentamycin. Pseudomonas was applied topically immediately after the creation of the burns in order to bypass the sterile conditions of the pathogen-free room and to mimic burn contamination that typically occurs in patients with burns.

Twenty guinea pigs were included in the study and were divided into three groups: Melolin-control group, soy protein dressing without antibiotic, and soy protein dressing with controlled release of gentamicin (Fig. 2). The closed (epithelialized) wound area and the open (non-epithelialized) wound area were traced on a transparent paper 12 days after creation of the burns. The degree of healing was calculated by the percentage of the epithelialized (closed) area on the 12th day compared to the total burn area on the 12th day. On the 12th day post-burn, epithelialization of the wounds that were dressed with Melolin covered 55% ± 15% of the burn area, the average closure of the soy protein group was 62% ± 13%, whereas the closure area of the soy protein group with gentamicin release reached 71% ± 11% (Fig. 3A). The difference in epithelialization and wound closure between the soy gentamicin group and the Melolin group was statistically significant (*p* = 0.04), while no significant difference was noted between the soy group and the Melolin group.

3.3. Wound contraction

Wound contraction was calculated as 1 minus the total wound area (epithelialized and non-epithelialized) divided by the original area subjected to burn. A significant reduction was found in the contraction rate between the soy treated groups (36% ± 8%, *p* < 0.02 for soy only, and 31% ± 10%, *p* < 0.005 for soy with gentamicin release) as compared to the Melolin group (51% ± 12%) (Fig. 3B).

3.4. Histological evaluation

Animals were sacrificed after 12 days, and 1 cm² biopsies were taken from the center of the wound and immediately fixed in phosphate buffered formalin. Two different observers
examined the biopsies that were stained with H&E (Fig. 4A–C), according to the abovementioned criteria. The gentamicin soy group was significantly superior to the other two groups ($p < 0.05$) (Fig. 4D): better epithelialization and neoangiogenesis, thicker dermis, fewer WBC, more follicles, and greater collagen content. The general scores differed significantly for the gentamicin release soy dressing (10.8 ± 1.4) versus the soy and the Melolin treatment groups (7.2 ± 1.7, 7.2 ± 1.2, respectively, $p < 0.05$). Wounds treated with controlled release of gentamicin obtained significantly higher histological scores than both controls after 12 days. This was mainly due to significant re-epithelialization of the wound ($p = 0.02$), better epidermis-to-dermis adherence ($p = 0.02$), amount of collagen ($p = 0.01$) and more skin appendages ($p = 0.03$). The differences in the amount of neoangiogenesis, WBC and the thickness of the dermis were not significant.

Furthermore, the soy dressing material was found to create good contact with the wound, and the soy itself did not cause any irritation to the animal’s skin.

## 4. Discussion

### 4.1. Gentamicin release profile

Commercial wound dressings that incorporate iodine (Iodosorb® by Smith & Nephew), chlorohexidine (Bipatch® by J&J)
Fig. 3 – (A) Percentage of wound healing at 12 days post-burn creation. (B) Wound contraction as percentage of total measured wound area (epithelialized and non-epithelialized) at 12 days post-burn creation relative to the wound area at the time of wound creation. Each dot represents the average of two burns on each animal (mean ± SD). Square (■) represents measurements of superimposed dots from two different animals. Triangle (▲) represents measurements of superimposed dots from three different animals. *Statistically significant as compared to Melolin treated group.

or most frequently silver ions (e.g., Acticoat® by Smith & Nephew, Actisorb® by J&J and Aquacel® by ConvaTec) as active agents are available in the market. Such dressings are designed to provide controlled release of the active agent through a slow but sustained release mechanism which helps avoid toxicity yet ensures delivery of a therapeutic dose to the wound. Despite frequent usage, there is growing evidence that silver is highly toxic to keratinocytes and fibroblasts and may delay burn wound healing if applied indiscriminately to healing tissue areas [30–32]. Furthermore, most of these dressing materials undergo frequent changes. Bioreabsorbable dressings may successfully address this shortcoming, since they do not need to be removed from the wound surface once they have fulfilled their role.

In our previous study of the soy protein wound dressings loaded with gentamicin we in vitro studied the antimicrobial effect of gentamicin release profiles on bacterial inhibition and also evaluated the cytotoxicity on fibroblast cells. Our main conclusions were that the gentamicin release from our soy films demonstrated great efficiency toward the relevant bacteria, which are abundant on human skin [20]. The films could effectively inhibit S. aureus and S. albus infections for at least two weeks and P. aeruginosa for three days. Filtered film extracts released during the first 24 h and from the 24–48 h showed no cytotoxic effect on the fibroblast cells, although the gentamicin quantities released were much higher than these of silver ion that are used in commercial wound dressings [20]. Based on these results, a selected soy protein film was used in the current animal study.

The in vitro gentamicin release profile from our selected film showed a moderate burst effect (54%) during the first 6 h, followed by a continuous decrease in release rates during the first two weeks. This was followed by a third stage of zero-order release (constant release rate) that lasted until the end of the experiment (8 weeks). It should be mentioned that the specimens maintained their integrity throughout the entire test period. The burst effect and release profile during the first two weeks were typical of diffusion-controlled systems. The third phase of constant release rate presumably involved some degradation of the soy protein matrix combined with diffusion of the remaining drug that was more firmly attached to the protein chains.

The obtained release profile could be beneficial for application as antibiotic-eluting wound dressings. During the first hours after being wounded, a relatively high drug release is essential to eliminate infections that may not have been eliminated during wound cleansing. Later, a continuous low release rate can keep the wound “infection free” for more than 2 weeks, which is the time usually taken for wound healing.

It should be noted that most of the gentamicin (75%) was released within the first week of the study, due to the hydrophilic nature of the antibiotic and the soy protein matrix. The hydrophilic nature of soy protein enables relatively rapid water intake, leading to full swelling of the matrix within a few hours of immersion. Unfavorable more rapid drug release rates have been reported in the literature for other antibiotic-eluting systems [33]. Controlling the release of antibiotics from these systems is challenging due to the hydrophilic nature of both the drug and the host polymer. In most cases the drug reservoir is depleted in less than 2 days, resulting in a very short antibacterial effect. Thus, our new antibiotic-eluting soy protein wound dressing is advantageous over other systems. The obtained gentamicin release profile can be explained by the fact that many antibiotic drugs bind proteins via van der Waals or ionic interactions. The bonded portion may act as a reservoir and be released more slowly than the unbound form. 0–30% of the adsorbed gentamicin binds to albumin [34]. Furthermore, gentamicin is a highly charged polycation (+3.5, pH 7.4) [35], whereas soy protein has many negatively charged
carboxyl groups. It is thus probable that some ionic bonding took place. Such a bonding mechanism has been shown before between deprotonated carboxyl groups of succinylated collagen and positive anion groups of the gentamicin molecule [36].

4.2. In vivo study

The post-burn end-point was chosen to be twelve days. At this stage, the wounds in all groups demonstrated epithelialization of more than half of the original wound area. The 3 study groups, with or without controlled release of gentamicin, were found to have 29–45% non-epithelialized areas and 31–50% contraction. A better epithelialization rate was observed for the soy with gentamycin dressing compared to soy without gentamycin and compared to the standard of care treatment of Melolin. Furthermore, a significant decrease in contraction rate was observed in the soy dressing with and without controlled release of gentamicin as compared to the Melolin dressing group (31% ± 10% (p < 0.005), 36% ± 8% (p < 0.02) and 51% ± 12, respectively). Wound contraction although it is an inherent part of a normal wound healing we would like to keep it to a minimum, since in humans it may lead to disfigurement of the skin and poor esthetic results. It may also lead to loss of the normal flexibility of the skin—a fixed deformity that entails a functional disability. The reduced effect of the contraction, which becomes a dominant mechanism when healing is delayed, is achieved by the significantly accelerated epithelialization as observed in the controlled release of gentamicin from the wound dressing group. Furthermore, it should be stressed that the soy dressing did not evoke a negative response due to its chemical, structural or physical properties. The controlled release of gentamicin dressing may have a systemic effect, and thus each animal received the same dressing from each treatment group to prevent cross contamination.

A clear benefit for the soy with gentamycin dressing was also found in the histological parameters compared to the control groups, meaning that the quality of healing was better when using the gentamicin dressing. Histological evaluation of the wounds showed superior organization of the epithelium and dermis in the gentamicin soy dressing compared to the control groups. The general scores differed significantly (10.8 ± 1.4, for the gentamicin soy dressing compared to the soy group, 7.2 ± 1.7, and the Melolin group, 7.2 ± 1.2, p < 0.05). The quality of the epidermis-dermis junction was better and there were more collagen fibers in the gentamicin dressing.
Another important finding in groups treated with controlled release of gentamicin was the dominance and good quality of the hair follicles and adnexa which are the source for re-epithelialization from within the burn area.

In conclusion, in vivo evaluation of the gentamicin-eluting soy protein dressing material in a contaminated wound demonstrated its advantage over the other investigated dressings. We consider the better qualities of the healed wound in the gentamycin soy dressing to be one of the most important advantages of this new dressing. The gold standard of local treatment with topical antibacterial agents, e.g. Silverol®[^1], is changed daily or twice-daily, which is time consuming and painful to the patient as well as less economical. Several of the currently used dressing materials that provide controlled release of silver ions as an antibacterial agent might have toxic effect on cells, which can delay wound healing. The current dressing material shows promising results, although it still needs evaluation in a clinical trial.

**Conflict of interest statement**

All the authors state no conflict of interest.

**Acknowledgements**

The authors are grateful to the Israel Science Foundation (ISF, grant no. 1055/11) for supporting this research. We would like to thank Solbar®[^3] (Ashdod, Israel) for kindly providing the soy protein isolate used in this study.

**REFERENCES**


