

***In vivo* efficacy of novel bioadhesives for closure of surgical incisions: Evaluation in a porcine model**

M. Foox¹, A. Keren^{2,3}, O. Pinkas⁴, E. Cohen³, N. Goldstein^{2,3}, A. Gilhar²,
M. Zilberman^{1,4*}, and Y. Ullmann^{2,3}

¹Dept. of Biomedical Engineering, Tel-Aviv University, Tel-Aviv 69978, Israel

²Faculty of Medicine, Technion—Israel Institute of Technology, Haifa 32000, Israel

³Dept. of Plastic Surgery and the Burn Unit, Rambam Health Care Campus, Haifa, Israel

⁴Dept. of Materials Science and Engineering, Tel-Aviv University, Tel-Aviv 69978, Israel

Surgical and traumatic wounds, including skin lacerations, comprise a major public health concern worldwide, with significant costs. In the last decades, the development of tissue bioadhesives as an alternative to sutures or staples for closing skin lacerations has intensified due to many advantages, including less pain and rapid application. Novel bioadhesives comprised of gelatin and alginate crosslinked with carbodiimide and N-hydroxysuccinimide were previously developed and studied by our research group. Based on our thorough *in vitro* study, four formulations of these novel bioadhesives were selected for the *in vivo* study in a porcine skin laceration model. Clinical, histological and immunohistochemical measurements were performed in order to evaluate the healing phase of wounds closed using our bioadhesives compared to stitches or Histoacryl® Blue. The results showed that incisions treated with formulations with a relatively low crosslinking agent content yielded better results in the healing phase compared to incisions treated with the basic formulation, i.e. no redness or scabs at the incisions area, similarly to the sutured incisions. The Histoacryl® Blue group was found in the least advanced phase and the treated incisions still had scabs. Histological and immunohistochemical analysis showed similar results of our bioadhesive formulations and stitches. In conclusion, our novel bioadhesives demonstrate a promising potential for use in wound closure applications. They were found to be superior to Histoacryl® Blue in most of the tests and presented an outcome similar to that obtained using stitches.

Keywords: gelatin, alginate, carbodiimide, clindamycin, bioadhesive, kaolin

1. Introduction

Every year, millions of people suffer traumatic wounds, including skin lacerations or surgical wounds which cause disruption of organs, connective tissue, muscles and tendons [1]. Sutures have been the traditional treatment for wound closure and bleeding control for many decades, due to their high tensile strength and low dehiscence. However, the inconvenience, painful and relatively slow handling, need for removal in some cases and concern over possible transmission of diseases through the use of needles are major disadvantages of suturing. Other techniques have been developed in order to address these issues, including the use of clips, staples, tapes, hemostasis agents and tissue bioadhesives [2, 3]. Tissue bioadhesives represent a group of

compounds that can be applied locally for a variety of indications, including bleeding control, wound closure, hemostasis, sealing air and body fluid leaks, repair of fistulas and aortic dissections, external fixation of devices and drug delivery [4]. An ideal bioadhesive should be easily and rapidly prepared, have rapid and strong bonding to the tissue, hemostatic properties and tissue healing regeneration characteristics, without interfering with the body's natural healing process. The most important requirements of bioadhesives are that they be safe, nontoxic, degradable and absorbable within the healing period, with minimal cytotoxic byproducts. They also need to be cost-effective [5–11].

Bioadhesives approved to date can be divided into several main families. Cyanoacrylates are a family of synthetic bioadhesives based on the exothermic polymerization of a liquid state alkyl-2-cyanoacrylate monomer in the presence of a nucleophile attack by weak bases such as water or

* Corresponding author

Prof. Meital Zilberman, e-mail: meitalz@eng.tau.ac.il

amines. The first cyanoacrylates approved for use were methyl-2-cyanoacrylate (Histoacryl® and Histoacryl® Blue) and ethyl-2-cyanoacrylate (Dermabond®). These cyanoacrylates are widely used in emergency rooms, dermatology, and plastic surgery, due to their high bonding strength to biological tissues, rapid curing time and ease of use. However, they have been limited to external or temporary applications due to the toxicity of their degradation byproducts, their low viscosity and high stiffness. All of the above can cause adhesion failure, tissue irritation, lipid membrane damage and inflammatory responses [1, 4, 15].

Another family of bioadhesives is based on poly(ethylene glycol) (PEG), a water-soluble, nontoxic, non-immunogenic, biocompatible, FDA-approved biomaterial that can be crosslinked by chemical crosslinking or by photo-crosslinking with photo-reactive elements such as acrylate groups, to form a hydrogel bioadhesive. Although PEG-based tissue bioadhesives have rapid gel formation, good adhesion to biological surfaces and are biocompatible, they have a swelling ratio of up to 400%, and require a relatively dry surface for good results [12].

Fibrins are among the most widely used tissue bioadhesives in clinical applications due to their fast curing, high biocompatibility and biodegradability. They consist of human-derived fibrinogen, factor XIII and other blood plasma proteins, which are activated by the addition of human or bovine thrombin and calcium chloride via the physiological cascade of coagulation. The resulting clot can decrease bleeding and leaking, contributes to repair of the vascular granulation tissue and is completely absorbed during wound healing [13]. However, fibrin bioadhesives pose risks and safety concerns, since they are prepared from human blood [4, 14–16]. There has been a concern for potential transmission of diseases, such as HIV and hepatitis A, B, and C [17]. Other disadvantages of fibrin glues include poor adhesion to the tissue, especially in high pressure bleeding, long preparation and rapid degradation [2].

Another family is protein-based bioadhesives that undergo crosslinking by formaldehyde, glutaraldehyde or carbodiimide while forming covalent bonds with the tissue surface. The main problem with these bioadhesives is the possible mutagenicity or carcinogenicity of the crosslinking agents. *In vitro* results showed that the biocompatibility of bioadhesives crosslinked with carbodiimide is significantly higher, whereas their bonding strength is significantly lower, compared to bioadhesives crosslinked with formaldehyde or glutaraldehyde [18].

Great effort is therefore currently invested in the development of a new generation of improved and ideal tissue bioadhesives. In order to combine biocompatibility with high bonding strength to the tissue and other desired properties mentioned above, we developed and studied novel tissue bioadhesive formulations based on a combination of gelatin and alginate, which are both natural polymers, crosslinked with N-ethyl-N-(3-dimethylaminopropyl) carbodi-

imide (EDC). This crosslinking agent is considered to be less toxic than other crosslinking agents used in the bioadhesives industry. Polymers from natural sources, such as gelatin and alginate, are currently in use in the field of regenerative medicine as implantable biomaterials, controlled-release carriers or scaffolds for tissue engineering, due to their similarity to the extracellular matrix and other polymers in the human body. Other advantages of the natural polymers are their excellent biocompatibility, biodegradability by enzymatic or hydrolytic mechanisms and unique mechanical properties. The effects of our bioadhesive formulations on the bonding strength, physical properties and cytotoxicity have recently been published [19]. Further studies in our lab succeeded in decreasing the crosslinker concentration by adding N-hydroxysuccinimide (NHS), without reducing the bonding strength to the tissue [20]. The release of bioactive agents such as pain relief medications and antibiotic drugs such as clindamycin was also successfully carried out in order to increase the bioadhesive's therapeutic effect [20–21]. Clindamycin, a lincosamide antibiotic, is used for treatment of a wide spectrum of diseases, including infections of the respiratory tract, skin and soft tissue infections, osteomyelitis, and gynecological infections [22]. A hemostatic agent such as kaolin, a contact pathway activator that initiates rapid clot formation in wounds, was included in the bioadhesive formulations in order to increase the adhesion ability in the hemorrhagic environment of the wound [23]. Four formulations were selected for the *in vivo* experiments described in this manuscript, based on our thorough *in vitro* study of these novel gelatin-alginate-EDC based bioadhesives [19–21, 23].

Porcine skin is anatomically and physiologically similar to human skin. For example, both have a thick epidermis and a similar dermis-epidermis thickness ratio [24]. Both show well-developed epithelial extensions that project into the underlying connective tissue (rete pegs), papillary bodies, similar dermal collagen and rich sub-dermal adipose tissue [25]. The size, orientation, and distribution of blood vessels in the porcine dermis are similar to blood vessels in human skin. Porcine and human skins are similar in terms of epidermal turnover time, types of keratinous proteins, and lipid composition. In addition, human and porcine skins heal through similar physiological processes. The pig is thus an excellent animal model for the assessment of post-trauma wound-healing agents intended for use in human wounds [24]. Such a model was therefore used in the current study to evaluate the effectiveness of our novel bioadhesives in the healing of skin lacerations.

2. Materials and methods

2.1. Materials

Gelatin “type A” from porcine skin (90–100 bloom), alginate sodium salt (viscosity ~250 cps, 2% (25°C)), N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydro-

chloride (EDC), N-hydroxysuccinimide (NHS), kaolin (K1512) and clindamycin hydrochloride were purchased from Sigma-Aldrich, Rehovot, Israel.

2.2. Preparation of tissue bioadhesives

Bioadhesive preparation was based on dissolving 200 mg/mL gelatin and 40 mg/mL alginate (Gel-Al) and the hemostatic agent (kaolin) or antibiotic drug (clindamycin hydrochloride) powders in distilled water, under heating up to 60°C. Various amounts of the crosslinking agents (EDC and NHS) were added to the Gel-Al solution containing the hemostatic agent or antibiotic drug just prior to the bioadhesive's use. All four studied formulations are presented in Table 1. Their *ex vivo* bonding strengths are also presented.

2.3. Ex vivo bonding strength in tension

Porcine skin (Kibbutz Lahav, Israel) was used as a soft tissue model. The porcine skin was cut into 2×2 cm² square-shaped pieces, and their epidermis side was firmly attached to metal testing holders with a matching surface area (all dimensions of the holders are specified elsewhere) [19]. One hundred and forty microliters of the bioadhesive were then spread uniformly on the dermis side of two porcine skin pieces (that were attached to the testing holders). These two porcine skin pieces were immediately attached by applying a 1.25 N load and were incubated at 37°C and 100% humidity. After 30 min, the bonding strength was measured in tension mode at room temperature using a 5500 Instron Universal Testing Machine (Instron Engineering Corp. Norwood, MA) and a 10 N load cell. The two parts of the joint were strained at a constant speed of 2 mm/min until separation was achieved. The mechanical testing procedure was inspired by the standard test method ASTM F-2258-03. The bonding strength was defined as the maximum strength in the stress-strain curve, measured by the Instron Merlin Software. Ten repetitions were carried out for each formulation.

2.4. Animal model

An animal experiment was conducted to evaluate the efficacy of our bioadhesive formulations compared to standard sutures and Histoacryl® Blue.

Animal handling was in accordance with national guidelines and was approved by the Technion Animal Care and Use Committee (IL1141112). The study was carried out on two large white juvenile domestic pigs (*Sus scrofa domestica*), weighing 60–65 kg. The animals were purchased from the Animal Research Institute, Kibbutz Lahav, Israel, and were housed in individual pens with an artificial 12 h light/dark cycle at constant temperature. The animals were acclimated for one week prior to the study and were fed a standard chow and water ad libitum.

2.5. Procedure and treatment groups

On the day of the experiment, the animals were anesthetized with an intramuscular injection of Ketamine (20 mg/kg) and ACP1 (1 mg/kg), followed by induction with propofol (5–7 mg/kg), maintenance after intubation with isoflurane 2% delivered by PPV+Fentanyl (5–10 mcg/kg/h). The ventral skin surface of the animals was shaved using an electric shaving machine followed by the application of an epilatory cream (Orna 19) to complete hair removal. The skin was then disinfected using a septal scrub (chlorhexidine disinfectant) and ethanol 70%.

Thirty 2-cm long full thickness incisions (the incisions went through the skin up to the subcutaneous plan), were made on animal number 1 by using a #10 blade [26]. The incisions were then randomly divided into five groups, i.e. six incisions per group. The groups included two control groups: 1) Stitches, 2) Histoacryl® Blue, and three experimental groups: 1) Basic, 2) Low EDC, and 3) Basic + Kaolin. Thirty-six 2-cm long incisions were made on animal number 2 by using a #10 blade. The incisions were then randomly divided into six groups, i.e. six incisions per group. The groups included two control groups: 1) Stitches, 2) Histoacryl® Blue, and four experimental groups: 1) Basic, 2) Low EDC, 3) Basic + Kaolin and 4) Low EDC + Clindamycin.

The first control group was sutured using interrupted 3-0 nylon, with 3 stitches on each incision and the second control group was glued using Histoacryl® Blue. The incisions of the experimental groups were treated in two steps: Gel-Al solutions (with or without additives) were spread into the cut wound using a syringe, followed by immediate

Table 1. The studied bioadhesive formulations and their bonding strength in tension

Bioadhesive formulation: Gelatin (mg/mL)-Alginate (mg/mL)- EDC (mg/mL)-NHS (mg/mL)- additive (% w/v)	EDC, mg/mL	NHS, mg/mL	Kaolin, % w/v	Clindamycin, % w/v	Bonding strength, kPa±STD
Basic (200-40-20)	20	–	–	–	13.9 ± 4.8
Low EDC (200-40-10-1)	10	1	–	–	20.0 ± 3.8
Basic + Kaolin (200-40-20-0-5% Kaolin)	20	–	5	–	15.8 ± 2.0
Low EDC + Clindamycin (200-40-10-1-3% Clindamycin)	10	1	–	3	18.0 ± 3.6

addition of a crosslinker solution. A Steri-Strip was placed on all incisions (control and experimental) and the incision areas were dressed with a hydrocolloid membrane (Granu-flex). All animals were treated with Tramadol (100 mg once a day) and Optalgin (500 mg twice a day) for three days. The animals were anesthetized at the endpoint, 14 days post-operation. The incisions were documented and 5 mm biopsies were taken from the center of the wound for histological and immunohistochemical analysis and immediately fixed in phosphate-buffered formalin. The healed skin sections were taken for *ex vivo* bonding strength measurements. The animals were then sacrificed [26].

2.6. Histological and immunohistochemical analysis

The skin biopsies were dehydrated with an increasing alcohol gradient, embedded in paraffin, and 5 μ m thick sections were cut using a Leica microtome. Slides were deparaffinized and hydrated with a decreasing alcohol gradient. Sections were then taken for standard hematoxylin and eosin (H&E) or Trichrome Stain (Gomori Kit) (Sigma-Aldrich, USA) analysis according to the manufacturer's instructions.

The antibodies for immunohistochemical staining included: anti-laminin antibody (Abcam, ab11575, Cambridge, UK), Ki-67 antibody (Zymed Laboratories, 7B11, San Francisco, CA), and anti- α SMA (smooth muscle actin) antibody (Abcam, ab5694, Cambridge, UK) [27–28]. The slides were deparaffinized and hydrated with a decreasing alcohol gradient and immersed in distilled water. For anti-laminin staining, antigen retrieval was performed using 1 mM TRIS-EDTA, pH 8, at 90°C for 13 min, followed by proteinase K digestion at 37°C for 10 min. For anti- α SMA and Ki-67 staining, antigen retrieval was performed using 1 mM TRIS-EDTA, pH 8, at 90°C for 20 min. The sections were then blocked with suitable serum for 30 min, followed by 14 h incubation at 4°C with the primary antibody. This was followed by incubation with an appropriate biotinylated secondary antibody, streptavidin-peroxidase conjugate, and S-(2-aminoethyl)-l-cysteine (AEC) as substrate (Histostain-SP kit; Zymed Laboratories, San Francisco, CA). Counterstaining was performed with hematoxylin and the slides were examined under a light microscope.

The evaluated criteria included: diffused collagen width, proliferation index, scar tissue formation and basement membrane integrity. The diffused collagen width was calculated as the average of 6–7 measurements from the neoepithelium to the dermis (measured under a light microscope). The integrity of the newly formed basement membrane was determined by evaluating the percentage of anti-laminin staining in the scar area [27–29]. The proliferation index of the epidermis was quantified in the scar area as the percentage of Ki-67-positive cells in order to measure keratinocyte activation. Scar formation was evaluated by counting anti- α SMA positive myofibroblasts in high power fields (the average of 5 fields). Anti- α SMA stain of

hair follicles and in smooth muscle of vessels was not counted in the analysis [27, 28]. All evaluations were performed by two observers in a single-blind trial under a light microscope.

For the histological evaluation, sections were observed and photographed under $\times 200$ and $\times 400$ magnification using an Olympus upright light microscope. Healing analysis was conducted in a double-blind manner by two separate evaluators using a quantitative grading system. The sections were evaluated based on structure and content.

2.7. Bonding force of the healed skin sections

Fourteen days post-surgery, the skin area containing closed incisions (sutured or attached using the bioadhesive) was harvested under general anesthesia, using a sterile no. 10 scalpel blade, and was cut into 5 \times 2 cm sections for the tensile force test using a 5500 Instron Universal Testing Machine with a 100 N load cell. The two parts of the skin samples were strained at a constant velocity of 2 mm/min until separation was achieved (Fig. 1). The bonding force was defined as the maximum strength in the stress-strain curve, measured by the Instron Merlin Software. At least 3 repetitions were carried out for each formulation.

2.8. Statistical analysis

All data were processed using the Excel software. Statistical comparison was evaluated by Student's t-test. A value of $p < 0.05$ was considered statistically significant.

3. Results and discussion

In the current study, we conducted an animal experiment to evaluate the efficacy of our bioadhesive formulations based on gelatin and alginate crosslinked with EDC compared to standard sutures and Histoacryl® Blue. As explained in the introduction section, porcine skin is ana-

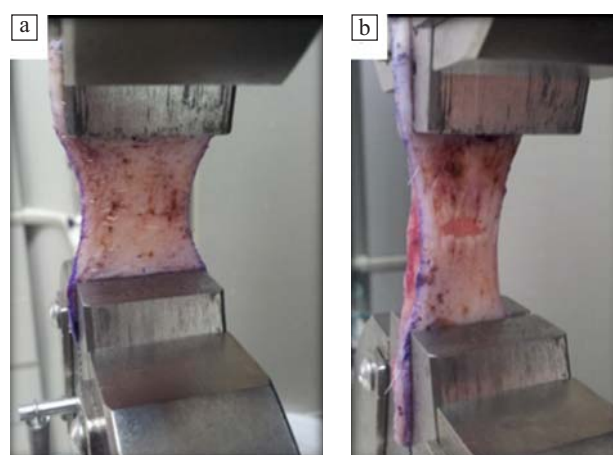


Fig. 1. *Ex vivo* bonding strength measurements. Skin sample, containing the incision area that was sutured, strained at a constant velocity of 2 mm/min: (a) until separation was achieved, (b) using an Instron Universal Testing Machine.

tomically and physiologically similar to human skin, and was therefore chosen as an animal model. Based on previous studies [19, 20, 23], four formulations with different concentrations of crosslinking agents and additives were chosen for this research (Table 1). NHS was added in order to increase the efficacy of the carbodiimide crosslinking reaction, whereas an antibiotic drug and a hemostatic agent were incorporated in order to increase the bioadhesives' therapeutic effect. The main results and their discussion are presented below.

3.1. Ex vivo bonding strength

High bonding strength to the tissue is crucial for tissue bioadhesives. The addition of kaolin, clindamycin or NHS increased the bonding strength of our basic formulation (Table 1). The incorporation of NHS in the crosslinking reaction enabled us to decrease the EDC concentration from 20 to 10 mg/mL, while the bonding strength increased from 13.9 ± 4.8 to 20.0 ± 3.8 . Addition of NHS to the crosslinking reaction of EDC is known to yield NHS-activated carboxylic acid groups, which are less susceptible to hydrolysis, prevent rearrangements, and shift the crosslinking reaction toward the creation of peptide bonds while reducing the side reactions [30]. The incorporation of kaolin increased the bonding strength from 13.9 ± 4.8 to 15.8 ± 2.0 . Pinkas et al. [23] suggested that kaolin can increase the cohesive strength of a bioadhesive via several mechanisms. First, kaolin's ability to absorb water and form a hydrate layer on its surface causes a decrease in water availability in the bioadhesive. It is known that free water disturbs the carbodiimide reaction [31]. Second, the negatively charged hydroxyl groups on kaolin's basal surface can form hydrogen bonds with the positive charges of amine groups in the gelatin, while the positive charges on the surface edge can interact with alginate's carboxylic groups [32–35]. The addition of clindamycin did not have a significant effect on the bonding strength. Clindamycin was chosen for use in this research for two reasons. First, contrary to other antibiotic drugs, it does not have primary amine or carboxyl groups. These groups can react with EDC, reduce the amount of EDC molecules available for the crosslinking of gelatin and alginate, and as a result decrease the crosslinking degree of the bioadhesive and its bonding strength [20]. Second, clindamycin release from the gelatin-alginate matrix was found to be relatively fast, controlled mainly by diffusion and swelling of the natural host polymers, and

highly effective against *Staphylococcus albus* and *Staphylococcus aureus* [20].

3.2. Wound closure

Overall, the animals tolerated the experimental procedures well and did not show signs of distress. The incisions were evaluated 14 days post-operation. Representative photographs of the incisions treated with various formulations of our bioadhesive and two control groups, stitches and Histoacryl® Blue, are presented in Fig. 2. Both the Histoacryl® Blue and the bioadhesive groups created good contact with the skin and no visible remains were left in the wound area at the endpoint.

Incisions were evaluated macroscopically 14 days post-operation. The healing process for each wound closed by application of the bioadhesives or by suturing progressed satisfactorily, without any apparent complications. The clinical observation showed that the incisions were at different levels of healing, while no infection was observed (Fig. 2). The stitches group was in the most progressive healing phase, while the Histoacryl® Blue group demonstrated scab formation and an overall relatively poor healing process. These results can be explained by the cytotoxic nature of Histoacryl® Blue, which stems from its degradation products, including cyanoacetates and formaldehyde, which can influence the healing process. Our bioadhesives are based on natural components such as gelatin and alginate, which are known to be biocompatible, and a crosslinker known to be less cytotoxic compared to other crosslinkers. When comparing between the bioadhesive groups, both Low EDC and Low EDC + Clindamycin formulations appeared to be at a more progressive healing phase, with no redness or scabs at the incision sites. Controlled release of clindamycin did not have a beneficial effect on wound closure. These results might have had a different outcome if the incisions were contaminated. One incision from the Basic formulation group was not properly glued and therefore did not heal properly.

3.3. Histological characterization

Histological study of the wound healing process is important for evaluating the efficacy of our novel bioadhesives compared to the control groups. A number of criteria determine the level of histopathological change. One of the most important criteria is collagen arrangement, since it plays a dominant role in maintaining the structural integrity of the

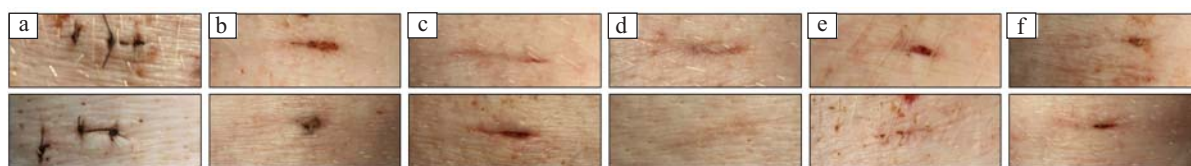


Fig. 2. Photograph of representative incisions from each group, 14 days post-operation. Control groups: stitches (a), Histoacryl® Blue (b). Our bioadhesive formulations: Basic (c), Low EDC (d), Basic+Kaolin (e), Low EDC + Clindamycin (f).

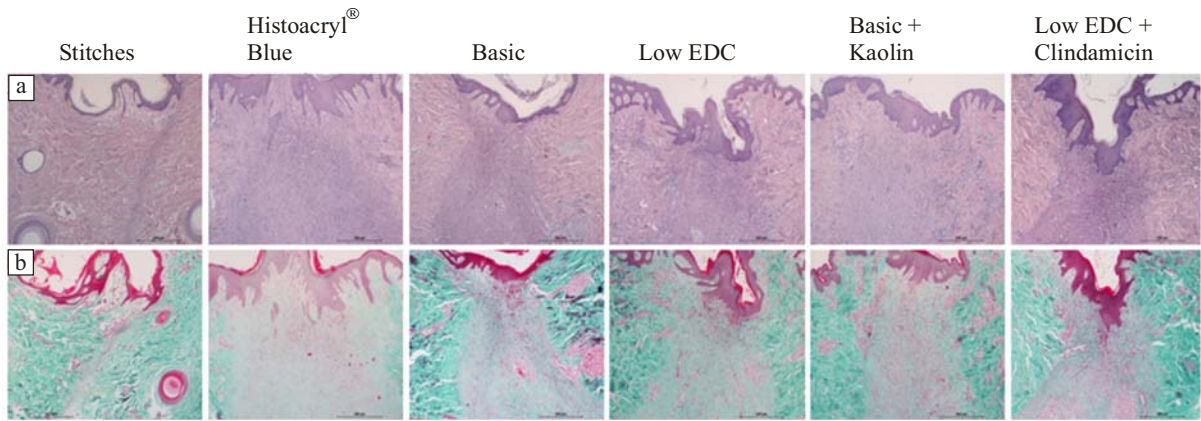


Fig. 3. Representative histological sections of the incisions from each group, 14 days post-operation: H&E staining (a), Trichrome staining for collagen fibers (b).

skin and provides strength to the tissue. H&E staining and Trichrome staining of histological sections of representative incisions taken upon sacrifice on day 14 are presented in Fig. 3. Trichrome Stain can clearly differentiate the important morphological keys for wound healing assessment, such as cytoplasm and muscle fibers (red color), cell nuclei (blue to black) and collagen fibers (aniline blue) [36]. The sutured wounds exhibited superior organization of the epithelium compared to the other groups. In addition, the collagen’s diffused width was significantly lower in the sutured group (Fig. 4) ($532 \pm 440 \mu\text{m}$) compared to the other groups. These results indicate a better healing process com-

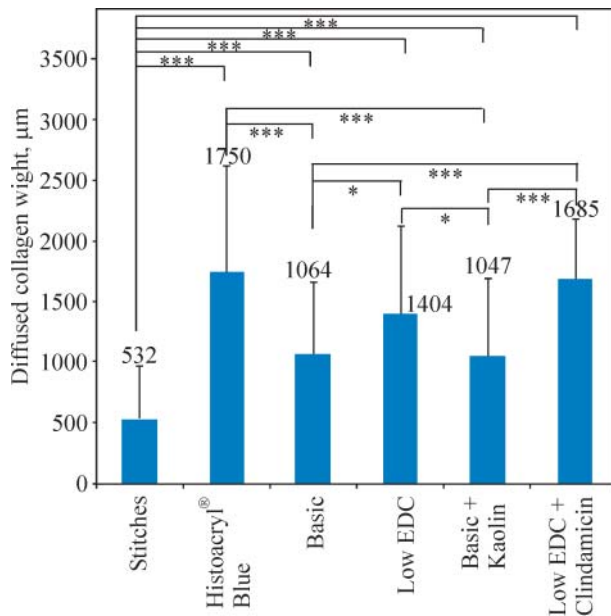


Fig. 4. Diffused collagen width of each group, calculated as the average of 6–7 measurements from the neoeplithelium to the dermis in the scar area, 14 days post-operation. Significant differences are indicated by: * $p \leq 0.05$ and *** $p \leq 0.001$.

pared to our tested bioadhesives, as seen in the clinical results. The Histoacryl® Blue group had the highest collagen diffused width ($1750 \pm 871 \mu\text{m}$), indicating a very slow healing process. These results can again be explained by Histoacryl® Blue’s cytotoxic effect on skin tissue. Among the test groups, the Basic formulations groups with or without kaolin had the lowest average diffused collagen width ($1064 \pm 599 \mu\text{m}$ and $1047 \pm 647 \mu\text{m}$, respectively), indicating that the high EDC concentration did not have a negative effect on collagen production.

3.4. Immunohistochemical characterization

Wound healing can be divided into four phases: homeostasis, inflammation (early and late), proliferation and remodeling. Immunohistochemistry can be used to understand the distribution and localization of differentially expressed proteins in the skin tissue, and can therefore be used to determine the specific wound healing phase. Ki-67 staining, a marker of the proliferative basal layer of the epidermis, was performed in order to determine the proliferation index of the epidermis and measure keratinocyte activation. It can be seen (Figs. 5 and 6) that the Low EDC + Clindamycin formulation and the stitches groups had the highest proliferation index (7.9 ± 2.9 and 6.5 ± 3.9 , respectively), indicating an advanced healing phase. The Histoacryl® Blue and the low EDC formulation groups exhibited the lowest proliferation index (3.6 ± 2.8 and 3.6 ± 2.4 , respectively).

Laminin staining was performed to determine the integrity of the newly formed basement membrane (a marker for epithelial integrity). The Basic + Kaolin formulation and the Low EDC + Clindamycin formulation demonstrated the highest basement membrane integrity in the scar area, indicating progressive healing (Fig. 7).

Scar formation was evaluated by counting anti- α SMA (smooth muscle actin) positive myofibroblasts in high power fields. Myofibroblasts represent key players in the physiological reconstruction of connective tissue after injury and

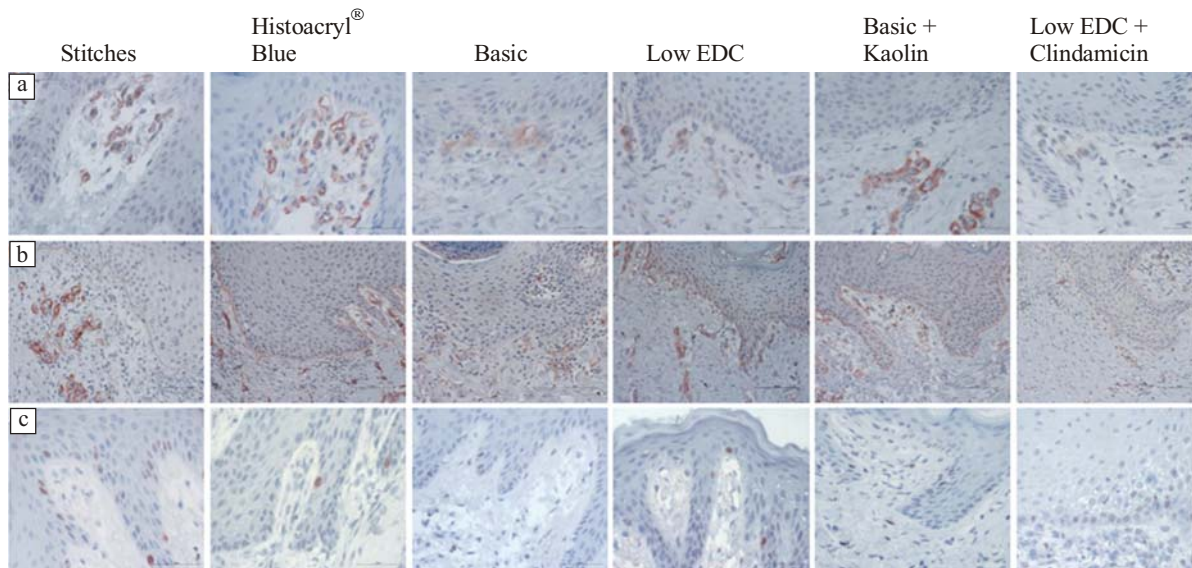


Fig. 5. Immunohistochemistry representative sections of the incisions from each group, 14 days post the operation: Anti- α SMA staining (a), Anti-Laminin staining (b), Ki-67 staining (c).

in generating the pathological tissue deformations that characterize fibrosis. No significant differences were found between the experimental groups and the control groups (Fig. 8).

3.5. Bonding force of the healed skin sections

The *in vivo* bonding force of the skin samples 14 days post-operation was weakest for the stitches group (17–18 N), and strongest for the Histoacryl® Blue group (37–39 N). Our novel bioadhesive formulations showed a wide range of bonding forces (17–31 N), which can be used for various applications.

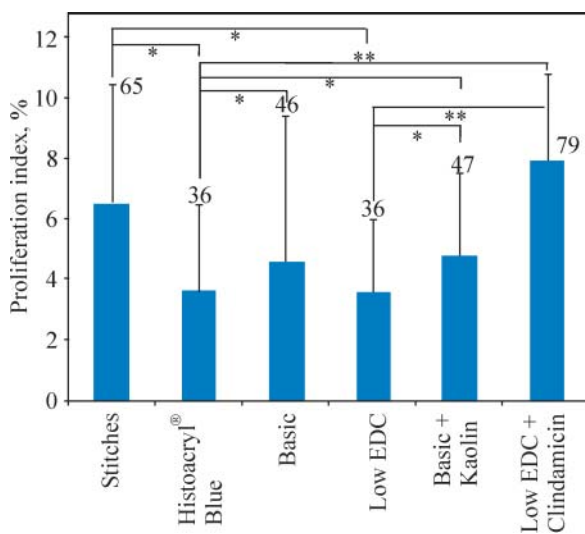


Fig. 6. Proliferation index of the epidermis (Ki-67-positive cells) in the scar area of each of the groups, 14 days post-operation. Significant differences are indicated by: * $p \leq 0.05$ and ** $p \leq 0.01$.

It should be noted that large variations in bonding force between similar samples were obtained, probably due to large differences in the size and thickness of the samples that were cut from the animals. The *in vivo* bonding force method can therefore be considered as a partially quantitative method, which provides a rough estimate of the strength of the healed tissue. The *in vivo* bonding force measurements of the healed skin sections that were treated with stitches or Histoacryl® Blue showed opposite results com-

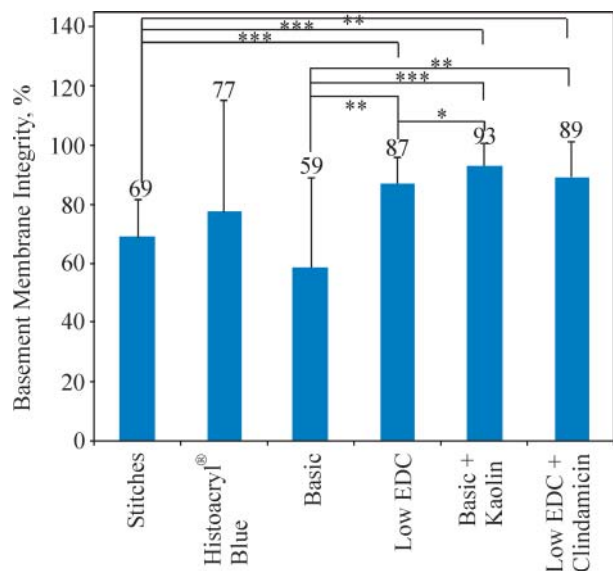


Fig. 7. Percentage of the anti-laminin stained basement membrane in the scar area of each group, 14 days post-operation. Significant differences are indicated by: * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$.

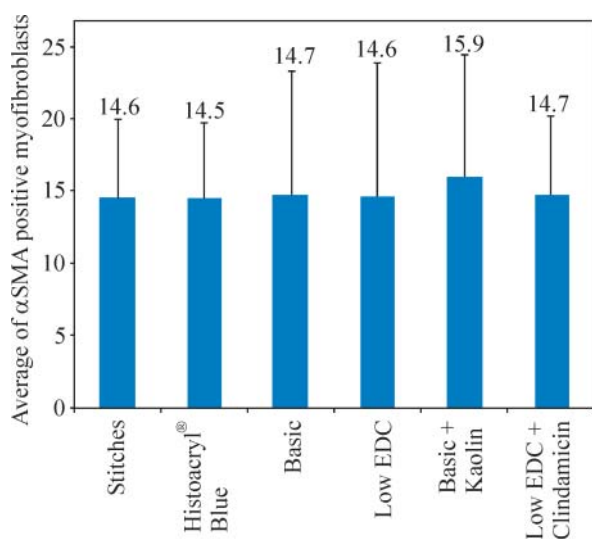


Fig. 8. Average of α SMA positive myofibroblasts in the scar formation of each group in high power fields ($n = 5$), 14 days post-operation. *Anti- α SMA stain of hair follicles and in smooth muscle of vessels was not counted.

pared to the clinical, histological and immunohistochemical analysis. Histoacryl[®] Blue is a synthetic bioadhesive known for its high bonding strength to biological tissues. However, it has been limited to external or temporary applications due to the toxicity of its degradation byproducts, their low viscosity and high stiffness.

4. Conclusions

In the current study, we conducted an animal experiment to evaluate the efficacy of our bioadhesive formulations based on gelatin and alginate crosslinked with carbodiimide (EDC), compared to standard sutures and Histoacryl[®] Blue. A porcine model for closure of surgical incisions was used. Clinical, histological and immunohistochemical measurements were performed in order to evaluate the healing phase of the wounds.

Two weeks post-surgery, the incisions were at different levels of healing, and no infection was observed. The sutured group was at the most advanced healing phase, showed superior organization of the epithelium and a significantly lower collagen diffused width compared to the other groups. When comparing between the bioadhesive groups, both Low EDC and Low EDC + Clindamycin formulations appeared to be at a more advanced healing phase, with no redness or scabs at the incision site, similarly to the sutured incisions. In contradistinction, the Histoacryl[®] Blue group was at the least advanced phase and the treated incisions still had scabs and showed the highest collagen diffused width, indicating a slow, and therefore less favorable, healing process.

The histological and immunohistochemical analysis showed similar results of our bioadhesive formulations com-

pared to the sutured group and higher bonding strength results. Unlike sutures, our novel bioadhesives do not require follow-up visits for removal, are less time consuming, less painful to the patient and offer a potentially valuable and economic approach for treating skin incisions.

In conclusion, our novel bioadhesives demonstrate a promising potential for use in wound closure applications. Further research is required in order to find the most efficient formulation.

Acknowledgements

The authors are grateful to the Kamin Program, at the Israel Ministry of Industry, Trade and Labor, for supporting this research.

References

- Bouten PJ, Zonjee M, Bender J, Yauw ST, van Goor H, van Hest JC, Hoogenboom R. The chemistry of tissue adhesive materials. *Progr Polym Sci.* 2014; 39: 1375–1405.
- Mehdizadeh M, Yang J. Design strategies and applications of tissue bioadhesives. *Macro Biosci.* 2013; 13: 271–288.
- Singer AJ, McClain SA. Development of a porcine incisional wound model and novel scarring scales. *Wound Rep Regen.* 2006; 14: 492–497.
- Reece TB, Maxey TS, Kron IL. A prospectus on tissue adhesives. *The American J Surg.* 2001; 182: S40–S44.
- Elsner JJ, Zilberman M. Antibiotic-eluting bioresorbable composite fibers for wound healing applications: Microstructure, drug delivery and mechanical properties. *Acta Biomater.* 2009; 5: 2872–2883.
- Lazovic G, Colic M, Grubor M, Jovanovic M. The Application of Collagen Sheet in Open Wound Healing. *Ann. Burns Fire Disasters.* 2005; 18: 151.
- Donkerwolcke M, Burny F, Muster D. Tissues and bone adhesives-historical aspects. *Biomater.* 1998; 19: 1461–1466.
- Heiss C, Kraus R, Schluckebier D, Stiller AC, Wenisch S, Schnetter R. Bone adhesives in trauma and orthopedic surgery. *Europ J Traum.* 2006; 32: 141–148.
- Mobley SR, Hilinski J, Toriumi DM. Surgical tissue adhesives. *Fac Plast Surg Clinics North America.* 2002; 10: 147–154.
- Otani Y, Tabata Y, Ikada Y. A new biological glue from gelatin and poly (L-glutamic acid). *J Biomed Mater Res.* 1996; 31: 157–166.
- Singer AJ, Hollander JE, Quinn JV. Evaluation and management of traumatic lacerations. *N Engl J Med.* 1997; 337: 1142–1148.
- Spotnitz WD, Burks S. Hemostats, sealants, and adhesives: components of the surgical toolbox. *Transfusion.* 2008; 48: 1502–1516.
- Brennan M. Fibrin glue. *Blood reviews.* 1991; 5: 240–244.
- Jackson MR. Fibrin sealants in surgical practice: An overview. *Am J Surg.* 2001; 182: 1S–7S.
- Raja STK, Thiruselvi T, Sailakshmi G, Ganesh S, Gnanamani A. Rejoining of cut wounds by engineered gelatin–keratin glue. *Biochim Biophys Acta.* 2013; 1830: 4030–4039.
- Valbonesi M. Fibrin glues of human origin. *Best Pract Res Clin Haematol.* 2006; 19: 191–203.

17. Quinn JV. *Tissue adhesives in clinical medicine*, second ed., BC Decker Inc, Hamilton, 2005.
18. Sung HW, Huang DM, Chang WH, Huang RN, Hsu JC. Evaluation of gelatin hydrogel crosslinked with various cross-linking agents as bioadhesives: in vitro study. *J Biomed Mater Res*. 1999; 46: 520–530.
19. Cohen B, Pinkas O, Foox M, Zilberman M. Gelatin-Alginate Novel Tissue Adhesives and their Formulation-Strength Effects. *Acta Biomater*. 2013; 9: 9004–9011.
20. Foox M, Raz-Pasteur A, Berdicevsky I, Krivoy N, Zilberman M. In vitro Microbial Inhibition, Bonding Strength and Cellular Response to Novel Gelatin-Alginate Antibiotic-Releasing Soft Tissue Adhesives. *Polymers Adv Tech*. 2014; 25: 516–524.
21. Cohen B, Shefy-Peleg A, Zilberman M. Novel Gelatin-Alginate Soft Tissue Adhesives Loaded with Drugs for Pain Management: Structure and Properties. *J Biomater Sci-Polymer Ed*. 2014; 25: 224–240.
22. Campbell IW, Hossack DJN, Munro JF. Absorption and urinary excretion of clindamycin palmitate in the elderly. *Curr Med Res Opin*. 1973; 1: 369–375.
23. Pinkas O, Zilberman M. Effect of hemostatic agents on properties of gelatin–alginate soft tissue adhesives. *J Biomater Sci-Polym Ed*. 2014; 25: 555–573.
24. Sullivan TP, Eaglstein WH, Davis SC, Mertz P. The pig as a model for human wound healing. *Wound Rep. Regeneration*. 2001; 9: 66–67.
25. Morris GM, Hopewell JW. Epidermal cell kinetics of the pig: a review. *Cell Proliferation*. 1990; 23: 271–282.
26. Zeplin PH, Schmidt K, Laske M, Ziegler UE. Comparison of various methods and materials for treatment of skin laceration by a 3-dimensional measuring technique in a pig experiment. *Ann Plast Surg*. 2007; 58: 566–572.
27. Loeffelbein DJ, Baumann C, Stoeckelhuber M, Hasler R, Mücke T, Steinsträßer L, Drecoll E, Wolff KD, Kesting MR. Amniotic membrane as part of a skin substitute for full-thickness wounds: an experimental evaluation in a porcine model. *J Biomed Mater Res B Appl Biomater*. 2012; 100: 1245–1256.
28. Hollander DA, Erli HJ, Theisen A, Falk S, Kreck T, Müller S. Standardized qualitative evaluation of scar tissue properties in an animal wound healing model. *Wound Repair Regen*. 2003; 11: 150–157.
29. Andree C, Reimer C, Page CP, Slama J, Stark BG, Eriksson E. Basement Membrane Formation during Wound Healing Is Dependent on Epidermal Transplants. *Plast Reconstr Surg*. 2001; 107: 97–104.
30. Timkovich R. Detection of the stable addition of carbodiimide to proteins. *Analyt Biochem*. 1977; 79: 135–143.
31. Nam K, Kimura T, Kishida A. Controlling coupling reaction of EDC and NHS for preparation of collagen gels using ethanol/water co-solvents. *Macromol Biosci*. 2008; 8: 32–37.
32. Alexandre M, Dubois P. Polymred-layered silicate nanocomposites: preparation, properties and uses of a new class of materials. *Mater Sci Eng*. 2000; 28: 1–63.
33. Sinharay S, Bousmina M. Biodegradable polymers and their layered silicate nanocomposites: in greening the 21st century materials world. *Progr Materials Sci*. 2005; 50: 962–1079.
34. Zheng JP, Li P, Ma YL, Yao KD. Gelatin/montmorillonite hybrid nanocomposite. I. Preparation and properties. *J Appl Polym Sci*. 2002; 86: 1189–1194.
35. Pavlidou S, Papaspyrides CD. A review on polymer-layered silicate nanocomposites. *Progr Polym Sci*. 2008; 33: 1119–1198.
36. Suvik A, Effendy AWM. The use of modified Masson's Trichrome Staining in collagen evaluation in wound healing study. *Malaysian J Vet Res*. 2012; 3: 39–47.