Supplementary data for the article:

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Supplementary material: In vivo test on wound healing and histological analysis

Materials and methods

In vivo test of dehydrogenate polymer in alginate hydrogel (DHP–Alg) on wounds

In vivo experiments were performed using male albino Wistar rats weighing 250 ± 20 g, reared and bred in the vivarium of the Institute of Physiology and Biochemistry, Faculty of Biology, University of Belgrade (Belgrade, Serbia). Experimental animals (two rats per cage) were housed under controlled temperature (21 ± 1 ºC) and lighting (12 h light:12 h darkness) conditions ad libitum.

The experiment was performed according to the rules for animal care proposed by the Serbian Laboratory Animal Science Association, a member of the Federation of European Laboratory Animal Science, and approved by the Ethics Committee of the Faculty of Biology, University of Belgrade. Rats were anaesthetised by ketamine + xylazine anaesthesia (80 mg/kg ketamine and 10 mg/kg xylazine mixed in a single syringe prior to administration). The dorsum was shaved with an electric hair clipper and was disinfected with 70% alcohol. Then, 2-cm linear skin incisions were made in the median plane beginning 3 cm after the cranial base to the below the inferior edge of the scapula using sterile No. 10 surgical scalpel blades. Rats were monitored under a heat lamp for 1 h postoperatively.

There were three experimental groups of animals: control (8 animals with sterile wounds without treatment); DHP−Alg (2 animals with sterile wounds treated with DHP−Alg); and antibiotic (2 animals with sterile wounds treated with chloramphenicol). The duration of treatment was 4 days. For DHP−Alg treatment, 500 μL of DHP−Alg was applied once a day at the same time. Concentrations of DHP and alginate were 0.1% and 2% (w/v), respectively. Ca^{2+} was added prior to applying the substances. Animals were euthanised at the 2nd or 6th day after treatment.

Histological and stereological analysis

Samples were taken from the control and DHP−Alg-treated animals after the wound healing period (2 days after treatment) and after the additional recovery period (6 days after treatment). Samples collected from the dorsal skin were fixed in 4% paraformaldehyde solution for 24 h at 4

°C, were dehydrated in increasing concentrations of ethanol and xylene and were embedded in paraffin (Histolab Products AB, Göteborg, Sweden). Each tissue paraffin block was serially sectioned at 5 μm thickness on a rotary microtome (RM2125RT; Leica, Glostrup, Denmark). The slices were placed on glass slides and were processed for routine haematoxylin–eosin (H&E) staining. Following staining, the sections were dehydrated in increasing concentrations of ethanol and xylene and cover slips were mounted with DPX (Sigma-Aldrich, USA). Analysis was performed in duplicate.

Results and discussion

After 4 days of wound treatment, wound healing was more expressed in wounds treated with DHP−Alg (faster epithelialisation) than in untreated wounds or those treated with chloramphenicol. DHP−Alg did not induce any inflammatory effect on the wounds or on the surrounding skin, in contrast to chloramphenicol that induced serious initial inflammation on the wound (Supplementary Fig. S2).

Two days after treatment (Supplementary Figs S2 and S3), the intense inflammatory reaction was dominant in the control group. On the surface, necrosis of skin tissue appeared as a consequence of mechanical damage. The beginning of formation of the demarcation line was observed under the tissue necrosis. Consequently, the cellular reaction of inflammatory phase was initiated (Supplementary Fig. S3A).

Examination of sections stained with H&E from the control animals after the recovery period (6th day after treatment) showed normal histological structure of the epidermis and dermis layers of the thin skin. The dermis contained sweat glands as well as hair follicles with the associated sebaceous glands that were surrounded by the arrector pili muscle (Supplementary Fig. S3B).

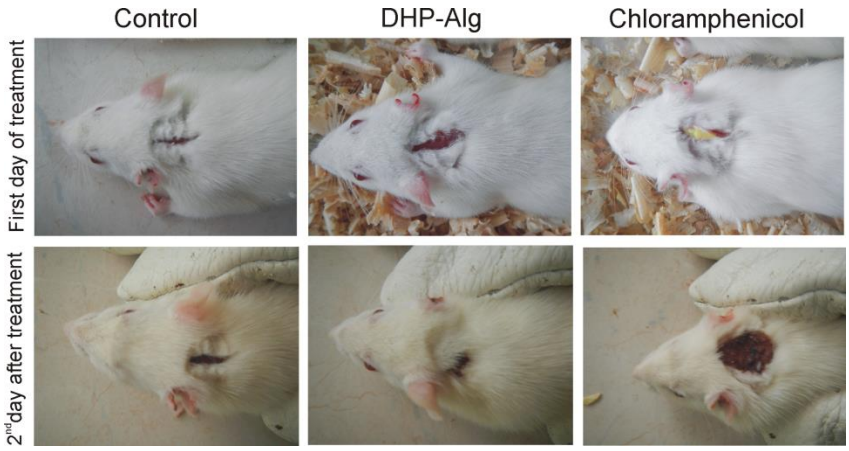
Regarding the DHP−Alg-treated animals, H&E-stained sections on the 2nd day after treatment showed disorganisation of the epidermal layers with an apparent increase in mitotic figures in the epidermal cells of different layers. Moreover, some of the keratinocytes appeared shrunken with widening of intercellular spaces and separation of cells. Some cells showed deeply stained pyknotic nuclei surrounded by pale clear vacuolated cytoplasm and others showed fragmented nuclei. The superficial layers showed a noticeable increase in the amount of basophilic granules

in cells of stratum granulosum and desquamation of the upper layer of the stratum corneum (Supplementary Fig. S3C).

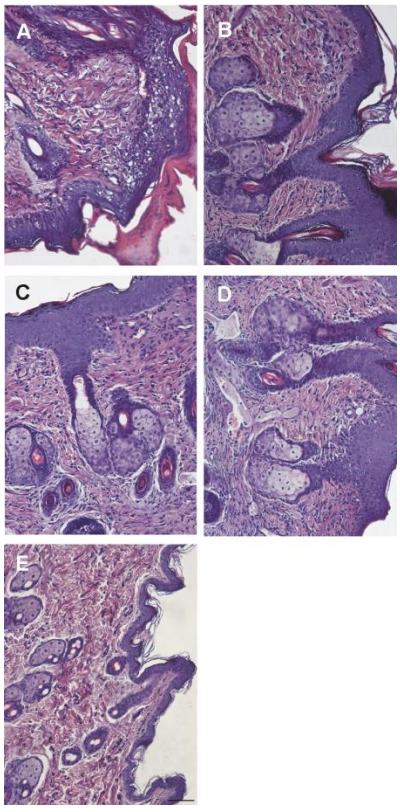
Examination of sections stained with H&E from the DHP−Alg-treated animals on the 6th day after treatment showed that most of the epidermal cells regained their normal appearance, whereas some cells were still vacuolated and some showed pyknotic nuclei. The intercellular spaces appeared slightly wider than normal. The stratum granulosum and stratum corneum appeared more or less normal (Supplementary Fig. S3D). In addition to epidermis regeneration, significantly increased angiogenesis and amount of collagen in the incisional space was observed in treated rats when compared with controls.

Histological analysis of sections obtained after treatment with antibiotic showed that the acute inflammatory process in all groups was in its final phase. Polymorphonuclear leukocytes were only randomly dispersed in the dermis and there was a moderate predominance of tissue macrophages. Keratinocytes migrated beneath the scab and completely bridged the whole incision (Supplementary Fig. S3E).

Histological sections from the DHP−Alg-treated animals post-wounding showed a significantly lower number of inflammatory cells than controls. In addition to epidermis regeneration, significantly increased angiogenesis and amount of collagen in the incisional space was observed in treated rats compared with controls. These results evidenced that the substance did not induce any damaging effects in both surface and deeper layers of the skin.



Supplementary Fig. S2. Examination of the effect of DHP–Alg on sterile wounds induced on the skin of laboratory rats. Control, DHP−Alg-treated and antibiotic-treated animals on the first day of treatment and 2 days after treatment are shown. DHP–Alg, dehydrogenate polymer in alginate hydrogel.



Supplementary Fig. S3. Histological sections of the wounds: (A,B) control animals on the 2nd and 6th days after treatment, respectively; (C,D) DHP−Alg-treated animals on the 2nd and 6th

day after treatment, respectively; and (E) antibiotic-treated animal. Scale bar: 50 μm. DHP–Alg, dehydrogenate polymer in alginate hydrogel.