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ORIGINAL PAPER

# Enhanced healing of skin wounds in ischemic rabbits using chitosan/hyaluronan/edaravone composite membranes: effects of laponite, carbon and silver-plated carbon nanofiber fillers

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**Abstract**
We examined the free-radical scavenging capacity of edaravone using the standard spectroscopic methods. Further, the impact of wound dressings composed of chitosan/hyaluronan with the addition of H atom and/or electron donoring edaravone in the absence and presence of laponite, C-nanofibers or silver-plated C-nanofibers was assessed on the healing of skin lacerations in ischemic ears of rabbits. The skin wound healing was compared with the untreated animals and animals, in which the wound was protected by a bicomponent chitosan/hyaluronan membranes. The in vitro results showed that edaravone in a dose-dependent manner was both free electron and H atom donor. The results of in vivo experiments showed that the addition of edaravone into chitosan/hyaluronan resulted in faster healing of skin wounds in ischemic rabbits. As proved also by histological investigation, the incorporation of laponite, C-nanofibers or silver-plated C-nanofibers into the composite membranes had significantly beneficial effects on skin wound healing.

**Keywords** Artificial skin · Free-radical scavenging capacity · Proliferation and remodelation phases of skin wounds · Wound dressings

## Introduction

Chitosan, a natural cationic polysaccharide consisting of (1 → 4)-2-amino-2-deoxy-β-D-glucan, is partially up to the fully deacetylated form of chitin. Sources of chitin include crustacean shells, fungi and algae cell walls, insect exoskeletons, and mollusk radulae (Matica et al. 2019; Sánchez-Machado et al. 2022). Chitosan has nontoxic, biocompatible, biodegradable, bioadhesive, antitumor, bioresorbable, and hemostatic properties, which allows its use in the pharmaceutical industry, especially in the control of the release of drugs, enzymes and vaccines, in tissue engineering, wound healing, bone regeneration, as well as biosensing/biomembrane manufacturing. Chitosan has been processed as powder, paste, gel, membranes, sponges, nanofibers, beads, microparticles, nanoparticles, and nanofibers (Kong et al. 2020; Sánchez-Machado et al. 2022). Chitosan facilitates wound healing and hemostasis and promotes cell proliferation and tissue regeneration. The degradation products, i.e., chitosan oligosaccharides, have excellent biological functions on tissue regeneration (Kong et al. 2020).

Hyaluronan (HA) is a linear polysaccharide consisting of alternate *N*-acetyl-D-glucosamine and D-glucuronic acid linked by β(1 → 3) and β(1 → 4) glycoside bonds, belonging to the glycosaminoglycans family. Due to its hydrophilicity and biocompatibility, HA is widely used as wound dressings in a form of films, hydrogels, fibers, non-woven fabrics and foams since it plays a crucial role in wound healing processes, being a major constituent of all vertebrates' connective tissue extracellular matrix, promoting the formation of a fibrin clot and the production of interleukins and pro-inflammatory cytokines. Moreover, polyanionic HA shows strong bacteriostatic activity as it helps to reduce bacterial adhesion and biofilm formation, especially HA of high molar mass (Della Sala et al. 2022).

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There are several papers reporting on the fabrication of chitosan/hyaluronic acid wound dressings (Deng et al. 2017; Thi-Phuong Nguyen et al. 2019; Garcia Garcia et al. 2020; Silvestro et al. 2020) and papers reporting on the fabrication of chitosan/hyaluronic acid wound treatment with the addition of a drug (Tamer et al. 2018a, b, c; 2020; Valachova et al. 2020, 2021a, b).

Edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one) is an antioxidant used to treat acute cerebral infarction in Japan. Edaravone has powerful free radical scavenging activity and can interact with both hydroxyl and peroxyl radicals to undergo oxidation. It is neuroprotective, promotes neovascularization and increases the mRNA expression of eNOS and protein in wounds of diabetic mice (Naito et al. 2014; Cha and Kim 2022). Concerning wound healing, edaravone protected $H_2O_2$-induced cellular injury via inhibiting early apoptosis and inflammation and also increasing angiogenesis of wounds in diabetic mice (Kim et al. 2020). Fan et al. (2019) showed that a low dose of edaravone-loaded nanocomposite hydrogel (0.1 mg) facilitated wound healing in diabetic mice. On the contrary, a high dose of edaravone (0.3 mg) could inhibit the healing.

Laponite is a synthetic hydrous sodium lithium magnesium silicate colloid (Lopez-Angulo et al. 2020). It becomes inert when in a physiological environment, while mixed with water, it changes to a clear gel with shear-thinning properties. The addition of laponite to chitosan or gelatin allows for the modification of their mechanical properties (Villalba-Rodríguez et al. 2021). Laponite is able to support cell adhesion, proliferation, differentiation as well as it has hemostatic properties (Zandi et al. 2021). As stated by Golafshan et al. (2017), laponite in the form of nanoplates — functioning as nano-fillers — has unique properties in wound healing.

In this research, this weakness was addressed by two different strategies consisting of formation of IPN hydrogels and nanohybrid hydrogels using laponite nanoplates as nano-fillers.

Carbon nanofibers (CNFs) are filaments of size in the range of $3-100$ nm in diameter constituted by stacked graphene layers with a certain orientation with respect to the fiber axis (Ruiz-Cornejo et al. 2020). They are highly hydrophobic, cost-effective, with good electrical, thermal and mechanical properties. Moreover, they have excellent physical and broad-spectrum antimicrobial properties. As stated by Salesa et al. (2021), CNFs were very effective in upregulating genes, which are involved in the defense mechanisms against oxidative stress and maintaining and repairing tissues by regulating cell adhesion, migration, differentiation, proliferation, morphogenesis, growth, and tissue development. For this reason, CNF and graphene fillers demonstrated a great potential in tissue engineering and wound healing (Kiedzierska et al. 2021).

Silver-plated carbon nanofiber fillers (Ag CNFs) are not unique materials; however, Ag CNF particles are classifiable as electrically conductive fillers (Cauchy et al. 2017). Yet, Ag CNF fillers have not been applied in wound healing so far (Dipl. Ing. Ivan Novák, CSc., personal communication).

The aim of this study was to examine in vitro radical scavenging capacity of edaravone using the ABTS and DPPH assays and in vivo effects of wound dressings (membranes) composed of chitosan and hyaluronan loaded with the drug edaravone itself and with the addition of bioactive fillers, namely laponite, C-nanofibers or silver-plated C-nanofibers.

## Experimental

### ABTS and DPPH assays – kinetics and determination of $IC_{50}$ values

The reaction kinetics between $ABTS^{\bullet+}$ and edaravone as well as between $DPP^{\bullet}$ and edaravone was studied by spectrophotometry as these methods were used also to determine the edaravone inhibition concentration at which 50% of the initial light beam is absorbed (Rapta et al. 2009; Valachová et al. 2015).

When applying the ABTS assay the stock aqueous solutions of edaravone (4, 1, and 0.4 mM) were used. The spectra were recorded at 734 nm within 10 min in 1-cm glass cuvette after mixing of the edaravone stock solution (50 µL) with the $ABTS^{\bullet+}$ solution (2 mL) using a UV/Vis-1800 spectrophotometer (Shimadzu, Japan).

When applying the DPPH assay the stock methanolic solutions of edaravone (4, 1, and 0.4 mM) were used. The spectra were recorded at 517 nm within 10 min in 1-cm glass cuvette after mixing of the edaravone stock solution (50 µL) with the $DPP^{\bullet}$ solution (2 mL) using a UV/Vis-1800 spectrophotometer (Shimadzu, Japan).

### Preparation of composite membranes

Chitosan (0.5 g) was dissolved in 20 mL of aqueous acetic acid (2%, v/v). Hyaluronan (50 mg) was dissolved overnight in 5 mL of water. Both solutions were then mixed together and loaded with 1 mL of the aqueous stock solution of edaravone (1.47 mg) itself and together with laponite (0.7 mg), CNFs (0.7 mg) or Ag CNFs (0.7 mg). Finally, 1 mL of glycerol as a plasticizer was added to the solution. This solution was then cast on a Petri dish, and the solvent was allowed to evaporate at room temperature for several days. The dry membrane, separated from the Petri dish, was rinsed for approx. 1 min in 1 M NaOH solution to remove traces of acetic acid. The membrane was then washed in distilled water, spread out and left to dry for several days at room temperature. Five types of membranes (5 per each type of

membrane) were prepared: 1/ Ch/HA membranes, 2/ Ch/HA membranes loaded with edaravone itself and with the addition of 3/ laponite, 4/ CNFs or 5/ Ag CNFs.

### Skin wound healing in ischemic rabbits

Experiments were approved by the State Veterinary and Food Administration in Bratislava, Slovakia (2908–3/20–220). Ischemic wounds on rabbits' ears were performed according to DiPietro's and Burns's method (DiPietro and Burn, 2003). Inside of each rabbit's ear, two lacerations with a size of ca. 1 × 1 cm and complete removal of skin tissue were performed. Rabbits were divided into six groups: 1/ group — control (wound was covered only with a bandage); 2/ group — rabbits' wounds covered with the Ch/HA membrane; and 3/ group — rabbits treated with Ch/HA/edaravone membrane; 4/ group — rabbits treated with Ch/HA/edaravone/laponite membrane; 5/ group — Ch/HA/edaravone/CNFs membranes; 6/ group Ch/HA/edaravone/Ag CNFs membranes. Post-operation animals underwent standard care. Animals were administered analgesics during the study. Rabbits were maintained individually in cages with an area of 4200 $cm^2$ in daily 12 h light–dark cycles. Animal wounds were covered with the membranes immediately after the primary treatment of wounds. Each membrane was moisturized in physiological solution (0.15 M aq. NaCl) and disinfected with ethanol (96%). Membranes were replaced with new ones after 3, 6, 9, and 12 days. The membranes were fastened to wounds with standard plasters, and all wounds were bandaged. The healing of skin wounds was evaluated by measuring of the wound area by planimetry. To statistically evaluate the healing of wounds two-way ANOVA test was carried out. Results are shown as the average and standard deviation for each group of animals.

On day 15, rabbits were sacrificed and the wound areas were harvested for histological examinations. The tissues were immediately fixed in 10% formaldehyde, then treated with conventional ethanol gradient dehydration and embedded in paraffin blocks. The tissues were sectioned into 3 µm thickness and stained with hematoxylin and eosin. The preparations were subsequently evaluated by an optical microscope (Olympus, Tokyo, Japan).

### Materials

**Chemicals** HA ($M_w$ = 1.69 MDa, $M_w/M_n$ = 1.63) was purchased from Lifecore Biomedical Inc., Chaska, MN, the USA. Chitosan (molar mass range 100–300 kDa) was obtained from ACROS Organics™, the USA. NaOH p.a., ethanol (96%), methanol (100%), formaldehyde solution (37 wt. % in $H_2O$), laponite-RD, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) (≥ 98%), 2,2-diphenyl-1-picrylhydrazyl (DPP), hematoxylin (high purity) and eosin (90%) were purchased from Sigma-Aldrich, St. Louis, the USA. Acetic acid p.a. was purchased from Centralchem, Bratislava, Slovakia. Edaravone was a gift of Dr. Veverka from Eurofins Bel Novamann, Bratislava, Slovakia. CNFs and Ag CNFs were gifts from Dr. Novák from Polymer Institute in Bratislava, Slovakia. Fifteen crossbred 6-month-old male rabbits HIL (2.5 ± 0.5 kg) from the Department of Toxicology and Breeding of Laboratory Animals at the Centre of Experimental Medicine in Dobra Voda, Slovakia, were used.

## Results and discussion

The results in Fig. 1, left showed high radical scavenging capacity of edaravone at concentration 100 µM (black). The percentage of unscavenged $ABTS^{\bullet+}$ was 6.1% even after 1 min of the measurement. A significantly lower effect of edaravone was shown at concentrations 25 and 10 µM. The

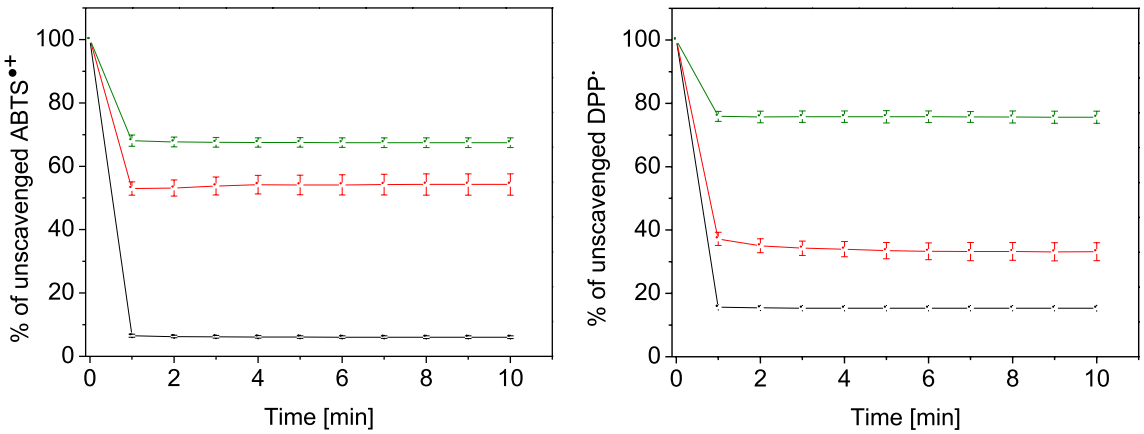


**Fig. 1** Percentages of unscavenged $ABTS^{\bullet+}$ (left) and $DPP^{\bullet}$ (right) using edaravone at concentrations 10 (green), 25 (red) and 100 µM (black) within 10 min

amounts of unscavenged $ABTS^{\bullet+}$ were 54.2 (red) and 67.4% (green), respectively, at 10 min.

Similarly, a high $DPP^{\bullet}$ scavenging activity (Fig. 1, right) of edaravone was reported at the concentration 100 µM (black). As shown, only 15.3% of $DPP^{\bullet}$ remained unscavenged. Edaravone at 25 and 10 µM concentrations did not scavenge 33.1% (red) and 75.7% (green) of the radical, respectively, at 10 min.

By using the ABTS and DPPH assays, we determined the $IC_{50}$ values $17 \pm 0.36$ µM and $17 \pm 1.23$ µM, respectively. Our determined $IC_{50}$ values are in the range of the values determined by other authors. The $IC_{50}$ values determined by the DPPH assay were 2.8 µM (Borges et al. 2013), $4.7 \pm 0.3$ µM (Tokumaru et al. 2018), $6.5 \pm 1.3$ µM (Takatsuka et al. 2021), 29.3 µM (Wang and Zhang, 2003). The $IC_{50}$ value determined by the ABTS assay was 2.01 µM (Borges et al. 2013), $24 \pm 5$ µM (Takatsuka et al. 2021).

While chitosan (positively charged) itself is able to form a film after drying of its slightly acidic solutions (usually in 2% aqueous acetic acid), HA (negatively charged) lacks such film-forming properties. Thus, when combining the two solutions in appropriate ratios, one may obtain a viscous solution, which after drying easily forms a thin film denoted as artificial skin. Such a film has much higher tear resistance compared to the film made only of the chitosan solution. After the addition of another component such as a drug or an antioxidant to the solution of those two polymers, we prepared a composite membrane, which is ready-to-be used for the treatment of, e.g., difficult-to-heal chronic skin wounds (Valachova and Soltes 2021b; Soltes et al. 2018, 2020). The third component was the drug edaravone itself and in the presence of bioactive fillers such as laponite, which acts as a molecular sieve (Powell, 2005) or CNFs, which are absorbents of polar and nonpolar substances (Chiang et al. 2021). Since this study was a pilot one, we examined the effects of edaravone at the concentration 1.47 mg/mL.

Results in Fig. 2 show the percentages of wound healing in ischemic rabbits within 15 days. The lowest percentage of wound healing was shown in untreated animals (gray). The values were in the range of 4% on day 3 up to 67% on day 15.

When treating the animals with Ch/HA membranes (red), the healing of rabbits´ skin wounds was more effective (27%) on day 3. During next days, the percentage of wound healing was increasing, and on the last day, the healing rate reached 98%.

The incorporation of edaravone into the two-component membrane (light blue) had a beneficial effect on wound healing, and on days 9 and 12, the healing was the fastest compared to the effects of other composite membranes. The addition of laponite into Ch/HA/edaravone (green) membrane had a positive effect on wound healing, especially on day 6, where the rate of healing was two times higher (67.8%) compared to the day 3 (33.1%). On other days, the

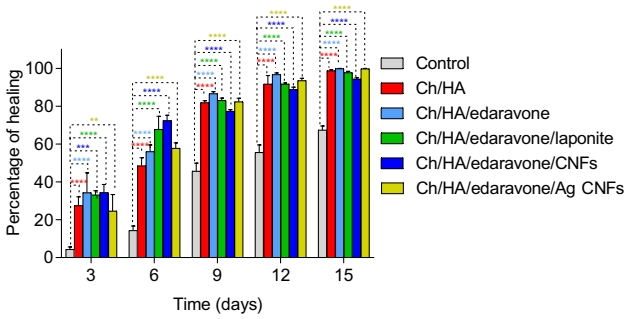


**Fig. 2** Percentages of healing skin wounds in ischemic rabbits within 15 days. ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$

rate of wound healing continued more slowly. Similarly, the effect of the addition of Ch/HA/edaravone loaded with CNFs (dark blue) was two times higher on day 6 (72.5%) compared to the day 3 (34.4%). However, on the next days, the wounds healed slowly. Compared to the effects of the membranes loaded with CNFs, the addition of Ag CNFs (yellow) had a positive effect on the rate of wound healing, which was evident from day 9. It is due to the presence of silver, which has high antibacterial properties, and it is widely used as antiseptic in wound healing (Pavlik et al. 2021).

At first, we confirmed that edaravone is a potential scavenger of reactive oxygen species inducing high molar mass hyaluronan degradation. Edaravone was shown to protect HA from $^{\bullet}OH$ and alkyloxy and alkylperoxy type-radical-induced degradation at low concentrations 6.25 and 62.5 µM (Tamer et al. 2018b).

Edaravone as a neuroprotective drug used in Japan was also effective in the treatment of skin wounds (Kim et al. 2020). For this reason, we examined the effects of CH/HA/edaravone membranes on skin wounds in rats, where these three-component composite membranes facilitated the healing of wounds compared to untreated animals and animals treated only with two-component Ch/HA membranes (Tamer et al. 2018b). Based on these results, we decided to assess the CH/HA/edaravone membranes on skin wounds of ischemic rabbits. Another option was to add to the previously examined three-component membranes laponite and CNFs, which is known to improve physico-chemical and biological properties (de Gonzaga et al. 2020; Salesa et al. 2021). CNFs were loaded with silver, which is known to have antibacterial properties (Pavlik et al. 2021).

Figure 3A displays that the tissue of the control group (untreated rabbits) was on day 15 in inflammation/proliferation phase. The tissue is granular and vascular maturating (*), with the prevailance of less amount of histocytes, leukocytes, hyperemic capillaries with perivascular bleeding and perpendicular distribution of fibroblasts. In rabbits treated with two-component Ch/HA membranes (Fig. 3B), the tissue was in a proliferative phase after 15 days. A maturating

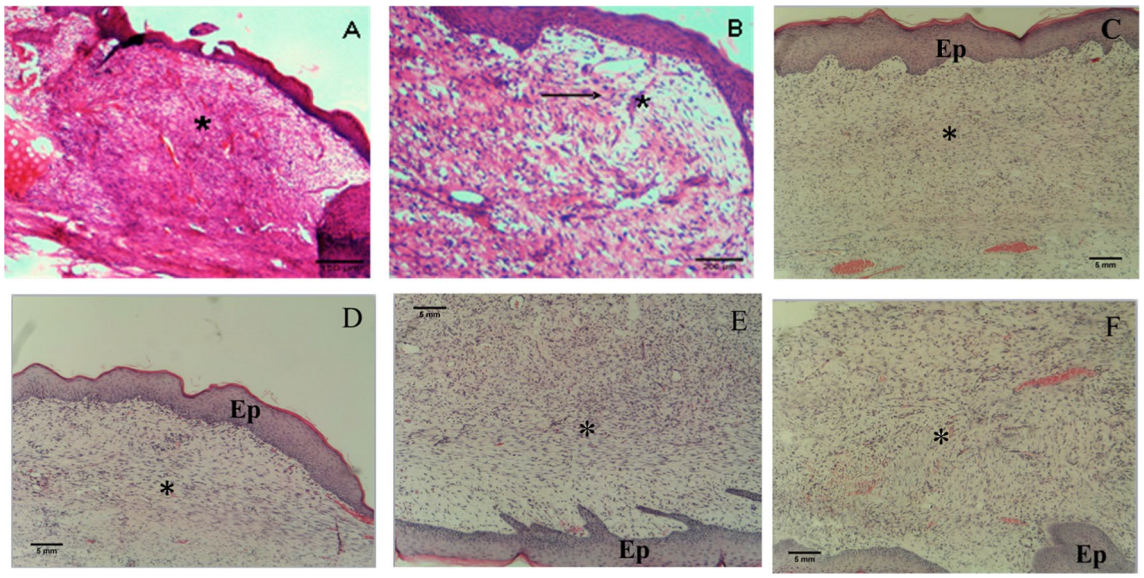


**Fig. 3** Ischemic rabbit wounds in ear tissues on day 15: Control (untreated animals, **A**), after treatment with two-component Ch/HA membranes **B**, after treatment with the Ch/HA composite membrane loaded with edaravone **C**, laponite/edaravone **D**, CNFs/edaravone **E**, Ag CNFs/edaravone. Ep: epidermis

granular tissue was shown, which was composed particularly of myxoid changes of the stroma leukocytes, plasmocytes, macrophages, and fibroblasts due to the presence of acid mucopolysaccharides. Moreover, there were activated fibroblasts with the formation of collagen fibers (→) and newly formed veins (*). Figure 3C illustrates a remodelation phase without inflammatory cellularization with fibroblasts (*) when the wound was treated with the Ch/HA composite membrane loaded with edaravone. Figure 3D and E displays the remodelation phase of wound healing using the Ch/HA composite membrane loaded with edaravone/laponite or with edaravone/CNFs, respectively. Figure 3F shows late hypocellular, hypovascular proliferative/remodelation phase (*) when the wound was treated with the Ch/HA composite membrane loaded with edaravone/Ag CNFs.

## Conclusions

As stated by Koizumi et al. (2006), edaravone treatment induces significant reduction in free radical precursors (OH, alkyloxy, alkylperoxy radicals). Based on our observation, the scavenging of OH, alkyloxy, alkylperoxy radicals by edaravone is linked with excellent H atom donoring properties, while the electron donor properties of edaravone (as proved by the DPPH assay) result in one electron donation to the counterpart substance (Watanabe et al. 2018).

According to the above-mentioned facts along with our results of in vivo experiments, we can conclude that edaravone added into chitosan/hyaluronan membranes led to more rapid healing of skin wounds in ischemic rabbits compared to untreated rabbits and rabbits treated only with chitosan/hyaluronan membranes. The addition of another component such as laponite, C-nanofibers or silver-plated C-had a positive effect on the treatment of skin wounds. These results were confirmed by histology.

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## Declarations

**Conflict of interest** The authors declare no conflict of interest.

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