Pharmacological Assessment of a Hialuronan Jelly to Wound Healing

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ABSTRACT
Hyaluronan or hyaluronic acid (HA) has been proposed as a therapeutic agent of wounds. Aim: Pre-clinical evaluation of possible HA-containing jelly therapeutics for veterinary and human use. Methods: 120 Sprague Dawley rats were tested for (5 60) or 7 (60) days. On the dorsum of each animal, we performed a wound with an 8 mm cutaneous biotome. Treatments were as follows: spontaneous control (C) allowing spontaneous recovery; placebo (P), positive control (C+) and therapy with 2 %, 4 % and 8 % of HA jelly. In each sacrifice 30 rats were used for planimetry studies and 30 rats for histological studies. Staining was performed with: haematoxylin- eosin (H/E) and Masson´s trichromic to evaluate the dermal reconstitution (DR) and epithelial migration (EM). Planimetry studies through a digital image processing estimated the re-epithelized area and other parameters. In all animals the biochemical indicators α amino and amino acids were measured. Results: Treatment with 4 % HA-jelly showed the highest response as evidenced by higher re-epithelization percentage, epithelial migrations and dermal reconstitution. Finally, aminoacids levels increased for all treatments with jelly in analyzed tissues. Conclusion: Results demonstrated that HA concentration in jelly formulations is critical to induce a wound healing response allowing a successful resolution. They identified that the formulation of jelly containing HA 4% is able to provide a strong and balanced healing therapeutic effect and it is a value therapeutic for healing wounds.

Keywords: Hyaluronan, wound-healing, assays, collagen, reepithelization.

Evaluación Farmacológica de una jalea de Hyaluronan en la Cicatrización de Heridas

RESUMEN
El Hyaluronan o ácido hialurónico (AH) se emplea como terapia en el proceso de cicatrización de heridas. Objetivo: el objetico de este estudio fue la evaluación preclínica de una jalea de AH para su uso veterinario y humano. Métodos: 120 ratas Sprague Dawley se trataron por 5 (60) y 7 días (60) después de provocar en el dorso de cada animal una herida con un biótomo de 8 mm. Los tratamientos fueron control espontaneo (C), placebo (P) control positivo (C+) y tratamientos con Jalea de AH al 2, 4 y 8 %. En cada sacrificio, 30 ratas se emplearon para la planimetría y 30 para la histología. Las muestras se colorearon con hematoxilina-eosina y con la tricrómica de Masson para evaluar la reconstitución dérmica (RD) y la migración epitelial (ME). La planimetría se hizo a través de un sistema de tratamiento de imágenes estimando el área reepitelizada y otros parámetros. Se midió en los animales los indicadores bioquímicos de α amino y los aminoácidos. Resultados: el tratamiento con la jalea de AH al 4 % mostró la mejor respuesta, esto se evidencia en el mayor porciento de reepitelización, la migración epitelial y reconstitución dérmica. Los aminoácidos se incrementaron en los tejidos analizados de los tratamientos con las jaleas. Conclusión: Los resultados demostraron que la concentración de AH en la jalea es fundamental para inducir la cicatrización de herida. Éstos identificaron que la jalea de AH al 4 % aporta un mejor y balanceado efecto terapéutico en la cicatrización de heridas.

Palabras claves: Hialuronan, cicatrización de heridas, ensayos, colágeno, reepitelización.

INTRODUCTION

Wound healing events occur in different steps as inflammation, proliferation and regeneration or resolution. Wound healing is a continuous biological process between the pathological state produced by inflammation and the regulation of cellular differentiation. The growth factors that control the development and the formation of new tissue contribute to repair [1-3]

Hyaluronan (HA) is a viscose polysaccharide that belongs to the glycosaminoglycans group (GAG). It is synthesized in the fibroblast membrane as well as smooth muscle, vascular epithelial and endothelial cells. HA is distributed in all extracellular spaces of the body, especially in the connective tissue. HA is involved in the wound healing response at various levels. After heat burns, increased plasmatic levels of HA has been observed [4] that contribute to the tissue damage repair process. Importantly, HA plasmatic concentrations decreased following considerable improvement of burned wounds [4, 5]. It contributes to the integrity of the fibro-connective tissue, promotes cell migration, proliferation and differentiation along the edges of the undamaged epithelium of wounds via regulation of epithelium and wound edge relation as well as epithelium and extracellular matrix interactions [6, 7]. Studies in experimental models have shown that the use of Hebermin (pharmaceutical product containing epidermal growth factor (EGF)) stimulates the synthesis of HA in the keratin forming cells of rats, where there is an elongation and migration of these cells and increased levels of HA in their peri-nuclear vesicles. Similar results have been observed in *in vitro* studies of wound healing [5, 8, 9].

Given its properties, HA has been used as a therapeutic agent. Thus, it has been demonstrated that HA accelerates healing since topical application of HA modulates its levels in the skin providing a beneficial effect in wound healing. These effects were described using tissue engineering and topical drug delivery of hydrogels from solubilised amnion membrane [9]. Besides, the use of membranes containing sterile etherified HA has been shown to induce a complete healing of wounds [9]. In addition, it has been reported that HA applied as a gel, is able to produce a complete healing in bilateral wounds of the mucose of the middle ear in animals [10-13]. For this reason, some authors consider the HA as a biopolymer with a wide therapeutic use, due to its versatility and ubiquity [14-16]

We have previously shown that jelly-based formulations containing different concentrations of HA have both pro-fibrogenic and immune regulatory functions in cell culture models [17]. With the aim to evaluate possible HA-containing jelly therapeutics for veterinary and human use, in the present study we assessed the therapeutic effects of HA-containing formulations in skin wounds of rats.

MATERIALS AND METHODS

Formulations

Healing Hyaluronic Acid -jelly at concentrations of 2 %, 4 % and 8 % (HA) was formulated for the first time at the Placental Histotherapy Center in Cuba. The manufacturing process consisted of separating residual umbilical cords of the human placentas obtained according to the National Re-collection Program, in Cuba [18].

Experimental Model

This study was performed on male Sprague Dawley rats weighing 200-250 g. Animal protocols were approved by the Ethics Committee for care and use of laboratory animals on Quality Control Drug Centre (QCDC), Havana, Cuba.

Rats were housed in individual cages in QCDC Vivarium. They were maintained in a 12 h light / 12 h dark cycle at room temperature (22 ± 2 ^{o}C), with food and water provided *ad libitum*.

The animals were anaesthetized with sodium pentobarbital (40 mg / kg/body weight (BW). The backs of the animals were depilated and cleaned with ethanol. An open excision of total thickness with a biotome of 8 mm diameter was made on the back of each rat under aseptic conditions, whose initial area measured 50.24 mm^2.

The animals were randomly divided into six groups. Each group had an equal number of 20 rats were given the different treatment. The six groups received the following treatments: (1) No therapy at all allowing a spontaneous evolution, (2) Placebo (carboxy-methyl-cellulose), (3)

Treatment with Aloe cream (10 % Aloe, CIDEM, Havana, Cuba) (positive control (C+)), (4) 2 % HA-jelly, (5) 4 % HA-jelly and (6) 8 % HA-jelly (formulation in Placental Histotherapy Centre, Havana, Cuba) [19]. The animals were treated topically, once per day during the study period.

On days 5 and 7 after treatment, ten animals in each group (60 animals each time) for the 120 total animals were randomly selected and sacrificed using cervical dislocation. Five rats were utilized in planimetry and histological studies for group. In total 30 samples were utilized each time for every study.

Planimetry Study

Sections of skin 5 mm wide were cut. The sections were immersed in 0.5 M sodium bromide and incubated for 24 hours at 37ºC. The epidermis was mechanically separated from the dermis. The dermal tissue was immersed in 6N HCl and the epidermis was placed on a labeled paper with the name of treatment. These samples were scanned using planimetry methods. The same samples were observed also by light microscopy. The imaging software (MADIG) [20] was used to determine the circularity (C), perimeter (P) and non-epithelization area (NEA) in the wound. The degree of re-epithelation (REA) and linear grow (LG) calculated using the following formulas:

$$REA = IA - NEA \ (IA = 50.24 \text{ mm2}) \quad (1)$$

$$LG = REA / aP \ (group) \quad (2)$$

Where IA is initial area (mm^2) and aP is the average perimeter of each group.

Statistical Evaluation

Simple ANOVA and multiple Range test (Start Graphic for Windows) were used to determine the statistical differences between groups. The confidence interval was determined to be $p < 0.05$.

Histological Evaluation

The samples for histological analysis were separated, fixed using 10% formalin, dehydrated through a graded alcohol series, cleared in xylene and embedded in paraffin wax. Serial sections of 7-10μm were cut and stained with haematoxylin and eosin, and with the Masson's Trichromic technique [21]. Afterwards, the samples were evaluated under a light microscope (Axiophot, Germany). The following criteria were developed to evaluate the classification of the evolution of epithelial migration and dermal reconstitution in the healing of open wounds in rats and were showed in tables 1 and 2.

Table 1. Criteria for the classification of the evolution of epithelial migration in the healing of open wounds in rats.

GRADE	EPITHELIAL MIGRATION
EMG I	Incomplete re-epithelization with presence or absence of the leukocytes in the coagulum of fibrin Poor projection of the epithelial edges of wound.
EMG II	Complete re-epithelization with presence of scab. Thin neo-epidermis under of the scab.
EMG III	Complete re-epithelization without scab, with moderate thickness in epithelium.

EMG: Epithelial Migration in Grade

Table 2. Criteria for the classification of the evolution of Dermal Reconstitution of open wounds in rats

GRADE	DERMAL RECONSTITUTION
DRG I	Incipient formation of collagen fibers focally distributed and disorganization. Moderate infiltration of macrophages and polymorphonuclears. Moderate angiogenesis.
DRG II	Partial reconstitution of the extracellular matrix with collagen fibers organized with a vertical disposition. Persistent discrete angiogenesis and few macrophages.
DRG III	Complete restitution of the extracellular matrix, with mature collagen fibers and organized horizontally. Some blood vessels may be found, or they may have collapsed due to the compression and presence of the around collagen fibers.

DRG: Dermal Reconstitution in Grade

RESULTS

Planimetry findings at the 5th and 7th days of treatment.

Tables 3A and 3B shows results of planimetry at 5th and 7th days of treatment, respectively.

Following 5 days of skin injury (Table 3A), those groups of animals representing negative controls (Groups 1 and 2) exhibited similar responses in all analyzed parameters ($p < 0.05$). On the other hand, both Aloe-treated (positive control) and HA-jelly treated rats showed a tendency for higher REA and LG and lower aP values when compared with groups 1 and 2. Interestingly, groups 4, 5, 6 showed significant changes as compared to groups representing negative controls ($p<0.05$). In addition, Aloe-treated and HA-jelly treated rats showed a tendency for lower C values when compared with groups 1 and 2. Particularly, groups 3 and 5 exhibited significant dismissed C values ($p < 0.05$).

Table 3A. Effects of various treatments on open wounds analyzed at 5th day following skin damage. Planimetry's results.

GROUPS	REA (± SD)		AP (± SD)	C (± SD)	LG (± SD)
	mm^2	%	mm	UA	mm
Control	35.44 (2.37) ^{a}	70.54	13.36 (1.09) $^{a'}$	53.10 (4.91) $^{a''}$	2.65 (0.18) $^{a'''}$
Placebo	35.04 (2,37) ^{a}	69.74	13.31 (1.09) $^{a'}$	52.70 (4.91) $^{a''}$	2.63 (0.18) $^{a'''}$
Positive control	40.99 (2.65) ^{ab}	81.58	10.37 (1.22) $^{ab'}$	33.20 (5.49) $^{b''}$	3.95 (0.20) $^{ab'''}$
2 % HA Jelly	43.24 (2.37) ^{b}	86.06	9.18 (1.09) $^{b'}$	45.28 (4.91) $^{ab''}$	4.70 (0.18) $^{b'''}$
4 % HA Jelly	44.74 (2.65) ^{b}	89.05	8.19 (1.22) $^{b'}$	34.92 (4.91) $^{b''}$	5.40 (0.20) $^{b'''}$
8 % HA Jelly	42.49 (2.65) ^{b}	84.57	9.67 (1.22) $^{b'}$	43.37 (5.49) $^{ab''}$	4.39 (0.20) $^{b'''}$

Shown are average values and standard deviations (SD) for each evaluated parameter. Degree of re-epithelation (REA), average perimeter (aP), circularity (C), and linear grow (LG). REA: b>a, Ap: b'>a', C: b''>a'', LG: b'''>a''', $p<0.05$; according to one way ANOVA followed by the Holm-Sidak post-test.

On the other way around, results of rats analyzed 7 days after skin injury illustrated that there was a tendency for greater REA values in different treated groups as compared to groups 1 and 2 (Table 3B). However, groups 4 and 5 displayed significant changes when matched with group 1 ($p<0.05$). Importantly, Aloe-treated and HA-jelly treated rats showed significant higher LG and lower aP and C values than those observed in groups 1 and 2 ($p<0.05$). It is worth noting that rats treated with the jelly containing 4% HA showed the greatest response ($p<0.05$).

Table 3B. Effects of various treatments on open wounds analyzed at 7th day following skin damage. Planimetry's results.

GROUPS	REA (± SD)		aP (± SD)	C (±SD)	LG (±SD)
	mm^2	%	mm	UA	mm
Control	45.24 (2.56) ^{a}	90.04	6.96 (1.63) $^{a'}$	35.68 (9.69) $^{a''}$	6.49 (0.34) $^{a'''}$
Placebo	46.64 (2.50) ^{ab}	92.83	5.96 (1.63) $^{a'}$	40.98 (9.69) $^{a''}$	7.82 (0.34) $^{a'''}$
Positive control	48.64 (2.50) ^{ab}	96.81	2.73 (1.63) $^{b'}$	14.14 (9.69) $^{b''}$	17.81 (0.34) $^{b'''}$
2 % HA Jelly	49.24 (2.62) ^{b}	98.00	2.71 (1.63) $^{b'}$	10.12 (10.83) $^{c''}$	18.16 (0.34) $^{b'''}$
4 % HA Jelly	49.84 (2.60) ^{b}	99.20	1.00 (1.63) $^{bc'}$	9.94 (9.69) $^{c''}$	49.73 (0.34) $^{c'''}$
8 % HA Jelly	47.64 (2.60) ^{ab}	94.82	3.34 (2.11) $^{b'}$	15.80 (12.51) $^{b''}$	14.24 (0.44) $^{b'''}$

Shown are average values and standard deviations (SD) for each evaluated parameter. Degree of re-epithelization (REA), average perimeter (aP), circularity (C), and linear grow (LG). **REA:** b>a, **aP:** c'>b'>a', **C:** c''>b''>a'', **LG:** c'''>b'''>a''', $p<0.05$; according to one way ANOVA followed by the Holm-Sidak post-test.

Histological findings of epithelial migration

Figure 1 shows epithelial migration rates observed at 5th and 7th days after skin damage. Groups 1, 2, 3 and 6 showed similar results ranging from 80 to 100% of EMG I representing open wounds at the 5th day of analysis. These groups also exhibited 60% of EMG I and 40% of EMG II

indicating a slight re-epithelization response 7 days following skin injury. Interestingly, however, groups 4 and 5 showed 40 and 60% of EMG II, respectively at the 5th day and even 20% of EMG III at the 7th day of study suggesting strong re-epithelization response.

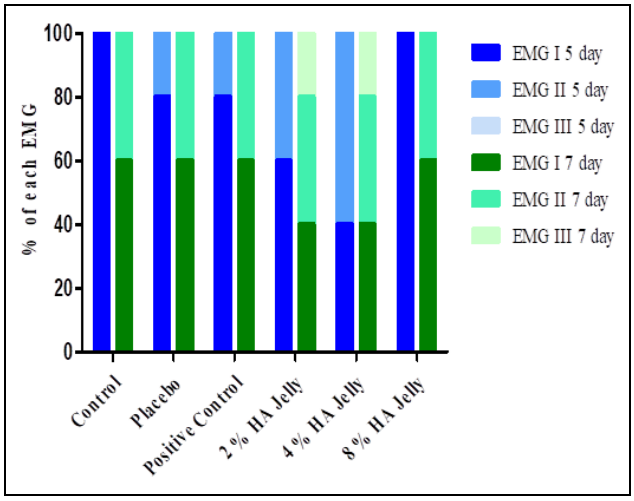

Fig. 1. Effect of HA in the epithelial migration process in open wounds expressed as % of each EMG. EMG (Epithelial Migration in Grade I, II, III) and HA (Hyaluronan).

Histological findings of Dermal Reconstitution

Figure 2 describes the dermal reconstitution process analyzed at 5th and 7th days after skin injury. At the 5th day of analysis, a similar behavior for groups 1, 2, 3 and 6 was observed, showing values ranging from 80 to 100 % of DRG I representing early stages of the wound healing response with low extracellular matrix formation. In addition, group 4 presented 40 % of DRG II which is consistent with moderate dermal reconstitution. Remarkably, animals treated with the formulation containing HA 4 % displayed 80 % of DRG II indicating strong and accelerated dermal reconstitution.

On the other hand, at the 7th day of analysis, negative control groups showed 60 % of DRG I and 40 % of DRG II. In contrast, groups 3, 4 and 6 considerably improved dermal reconstitution exhibiting only 20 % of DRG I, and even groups 4 and 6 revealed 20 % of DRG III. Moreover, the group of animals receiving the HA 4 % Jelly showed 40 % of DRG II and 60 % of DRG III, thus supporting enhanced wound healing response.

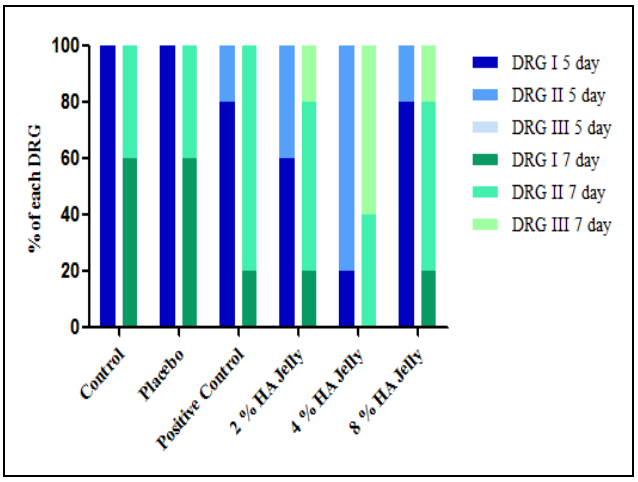

Fig. 2. Effect of HA in the dermal reconstitution process in Open Wounds expressed as % of each DRG. DRG (Dermal Reconstitution in Grade I, II, III) and HA (Hyaluronan).

The HA Jelly had good tissue recuperation, but is higher in the jelly at 4 %. Representative images of the histological effects on animals receiving the HA 4 % Jelly as compared to group 1 are shown in figure 3.

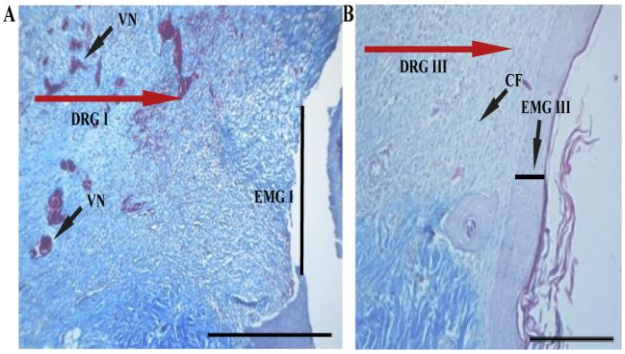

Fig. 3. Representative skin histology images of a negative control (200X) (A) and treatments with the 4% HA Jelly (100X) (B) groups. Masson´s Trichromic Staining. A: Predominant EMG I with not epithelial migration to the edge and DRG I predominance with inflammatory cells and new vessels blood (VN) formation. B: Predominant EMG III epithelium closed wound with moderate thickness and RDG III with structured collagen fibers organized horizontally (CF) (Bar = 200µm).

They demonstrated the therapeutic effect of the formulation containing HA 4 %. Note that while the

image from the negative control group showed almost no collagen fiber formation with predominance of granulation tissue and open wound, that of the group 5 exhibits full reconstitution dermis with organized collagen fibers and complete re-epithelization, this is similar to effect in MEG and DRG (figure 1 and 2).

Biochemical findings

Next the amount of aminoacids per area of analyzed tissue was calculated as an indicator of protein levels in these samples. Particularly, the presence of N-α NH2 groups represents total amino acids per area. A slight increase in the amount of N-α NH2/area was observed in group 6 relative to group 1(1.5 times) after 5 days of treatment. However, following 7 days of dermal injury, group 5 showed a strong increase of N-α NH2/area relative to group 1 (2.74 times) while in group 6, N-α NH2/area increased 2.26 times relative to group 1 (figure 4).

On the other hand, table 4 represents relative amounts of specific amino acids per area of analyzed tissue. They represent aminoacids that are enriched in collagens. Results showed that these aminoacids increased mainly in groups 5 and 6 at all times suggesting augmented cellular processes involved in protein synthesis including the formation of new extracellular matrix.

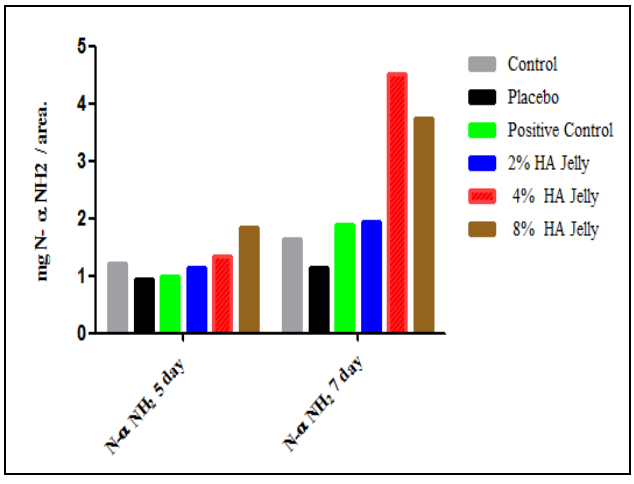

Fig. 4. N-α NH2 quantification at 5th and 7th days after treatments.

Table 4. Specific amino acids in the neo-formed matrix of rats' skins, measured 5 and 7 days after treatment (relative area of chromatograms)

Groups	OH-PRO		PROLINE		GLYCINE	
	5th	7th	5th	7th	5th	7th
1	3.46	7.52	8.77	10.38	107.42	125.60
2	3.98	7.24	11.04	29.68	104.91	120.91
3	4.95	6.37	12.4	15.94	198.3	206.02
4	4.15	4.61	16.08	29.33	121.45	138.46
5	9.34	10.33	30.74	153.75	191.85	309.87
6	8.42	13.39	42.16	163.68	194.33	401.76

DISCUSSION

The approach and parameters used in this study have been previously shown to be useful to evaluate the healing capacity of therapeutic candidates [24, 25]. Results from this work showed the therapeutic value of the Jelly formulation containing 4 % of HA. Accordingly, planimetry, histological and biochemical evidences support these findings. HA has been described to accelerate the healing process through the formation of granulation tissue, its substitution by well aligned collagen fibres and angiogenesis [23, 26, 27]. A possible explanation for our results may be related to the viscosity of the formulations evaluated. The jelly formulation containing 4 % of HA is less viscous than the one with HA 8 %. Therefore, we noted that the HA 4 % jelly showed better adhesion and permanence in skin than the HA 8 % jelly. Alternatively, the application of jelly formulation containing 8 % of HA may induce excessive inflammatory responses that may affect subsequent phases of the wound healing response including fibrogenesis and resolution. In fact, increased levels of HA in serum have been associated with several inflammatory skin diseases, such as psoriasis [28, 29]. Our hypothesis is supported by the analysis of aminoacids levels in wounded tissues. This study showed a tendency for increased aminoacids levels in tissues treated with the HA 8 % jelly. While levels of aminoacids may be related to fibrogenesis and the formation of a fibrous collagen matrix [30-36], they may also be associated with other cellular processes involving protein synthesis such as the inflammatory response,

angiogenesis and cell proliferation [23, 37-41]. Importantly, these are hallmarks of HA-induced biological responses.

HA contributes to the wound healing response through various ways. On one hand, low molecular weight HA has been shown to be release soon after skin injury contributing to the induction of interleukin 8 (IL8) in endothelial cells and the initial inflammatory step of the wound healing response [35, 37, 40, 42]. Thus, HA accumulates in the wounded tissue during the early inflammatory phase where it interacts with CD44 to further promote the release of pro-inflammatory cytokines. This response enhances cell infiltration and angiogenesis, as well as fibroblasts and keratinocytes proliferation and cell migration [23, 37-41]. HA participates in additional cellular functions such as cell aggregation, retention of pericellular matrix and matrix-cell signalling [43-45]. On the other hand, HA has been described as a free radical scavenger regulating the oxidative response induces by the cutaneous injury [38, 46, 47]. Consequently, the high molecular weight HA has been shown to restrict the movement of reactive oxygen species [46, 48]. In addition, enzymatic digestion of HA and spectroscopy analysis indicated the presence of a double bond in the D-Glucoronic acid unit which can form a complex with reactive oxygen species reducing the toxicity of radicals [38, 46, 47]. These antioxidant properties of HA appear to moderate the inflammation, to prevent oxygen free radical damage of granulation tissue and to promote the resolution phase of the wound healing response [38, 45].

Thus, it is possible to suggest that the HA 8 % jelly predominantly induced a deregulated wound healing response taking longer to close the wounds. Treatment with the HA 4 % jelly, on the other hand, induced an accelerated and balanced curative response possibly involving the strong antioxidant properties of HA. This is supported by the fact that both HA 4 % and HA 8 % formulations similarly increased the levels OH-proline at 5 days of treatment but only animals receiving the HA 4 % jelly showed closed wounds at this time. In addition, just rats treated with the HA 8 % jelly but not with the HA 4 % jelly, continued augmenting OH-proline at 7 days of treatment. As OH-proline is marker of collagen production [34, 49-51], these findings indicate that the HA 8 % jelly stimulated a deregulated response as compared to the HA 4 %-containing formulation. Notably, these *in vivo* results are supported by our previous reported effects of HA-containing formulations in cell culture showing strong immunoregulatory properties of the HA 4 % jelly [17]. Particularly, the formulation containing HA 4 % induced lower levels of proinflammatory TNF-α and higher levels of regulatory IFN-α [17, 52]. Regulatory functions of the HA 4 % jelly may also be related to its anti-apoptotic effects, stimulatory or inhibitory actions on TGF-β production, and induction of immunoregulatory IL-10 [17].

By providing a balance between the stimulating properties of HA on the initial and perpetuation phases of the wound healing response and the regulating anti-inflammatory and antioxidant effects promoting resolution, the HA 4 % jelly is able to provide a strong healing therapeutic effect. Further studies are necessary to prove the value of this prominent formulation in humans.

CONCLUSIONS

Results shown in this study constitute a further step in development and evaluation of a value therapeutic for healing wounds in animals and humans. They demonstrated that HA concentration in jelly formulations is critical to induce a wound healing response allowing a successful resolution. They identified that the formulation of jelly containing HA 4 % is able to provide a strong and balanced healing therapeutic effect.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interests regarding the publication of this paper

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