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Jacobs Journal of Clinical Trials

Research article

# Hyaluronic Acid and Silver Sulfadiazine Combination: Evaluation in a Second Degree Burns Rat Model

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*Received: 07-13-2016*

*Accepted: 10-18-2016*

*Published: 11-01-2016*



## Abstract

**Objective:** Assessing the benefit of a hyaluronic acid-silver sulfadiazine combination versus a silver sulfadiazine formulation in a rat model.

**Methods:** A deep second degree burn was induced on the lower and upper back of 59 rats using a standardized severe burning procedure. The burn was daily dressed and responses to both treatments were assessed until complete wound closure. Macroscopic (inflammation, moisture environment, epithelization and retraction) and microscopic (dermis formation, blood vessels, collagen reorganization, early annexes) parameters were assessed.

**Results:** Hyaluronic acid silver sulfadiazine combination decreased and almost prevented apparition and persistence of exudate (13.6% ± 4.5 vs 81.4% ± 5.1 of treated wounds; 1.63 ± 0.26 vs 7.19 ± 0.63 days) and maceration events (18.6% ± 5.1 vs 98.3% ± 1.7 of treated wounds; 2.36 ± 0.64 vs 12.76 ± 0.74 days). While no significant difference was found in terms of complete epithelization, the later appeared faster using hyaluronic acid silver sulfadiazine combination (25% of wound epithelization was reached at 19.58 ± 0.48 vs 22.40 ± 0.65 days and 50 % of wound epithelization at 30.91 ± 0.94 vs 35.44 ± 1.41 days). Wound retraction was also achieved significantly faster when using hyaluronic acid silver sulfadiazine combination (25% was reached at 13.89 ± 1.19 vs 29.72 ± 1.28 days and 50% at 35.48 ± 1.08 vs 42.09 ± 0.92 days).

**Conclusions:** The hyaluronic acid silver sulfadiazine combination appears well indicated for the treatment of a deep second degree burn, probably by creating an optimal and favourable environment for an efficient wound healing process.

**Keywords:** Second Degree Burn; Hyaluronic Acid; Silver Sulfadiazine

**Abbreviations**

AFA: Alcohol, Formalin and Acetic Acid;

HA: Hyaluronic Acid;

SEM: Standard Error of the Mean;

SSD: Silver Sulfadiazine;

HA-SSD: Hyaluronic Acid and Silver Sulfadiazine combination

## Introduction

Thermal burn is one of the most common injury worldwide [1,2]. Severity is based on burn depth assessment that will then determine the appropriate treatment. Classification as first, second and third degree burn is closely depending on the severity of epidermis and dermis damage [3-5]. The major complication for second and third degree burn remains wound infections which are a significant source of morbidity and mortality [6,7]. A moisturizing ointment will be commonly used for a first degree burn while surgical treatment will be necessary for third degree burn [8]. Second degree burns represent 50-63% of burn admitted patients in emergency department [9,10] and are usually treated using topical antimicrobial [8,11,12]. Among them, the gold standard is a formulation containing 1% silver sulfadiazine (SSD). This treatment combines a high bactericidal activity and a low toxicity that facilitates wound healing [11,13]. However, SSD still remains controversial as a delay in wound healing is observed when compared to non-bactericidal dressings [14-17].

Hyaluronic acid (HA), a non-sulphated glycosaminoglycan and a major component of the dermal extracellular matrix, has widely demonstrated beneficial effects in wound healing [18,19]. Indeed, HA regulates inflammation, enhances fibroblasts and keratinocytes proliferation, and increases collagen synthesis [20-22]. HA also ameliorates defence against microorganisms by inducing keratinocytes β-defensin 2 [23]. Nevertheless, no clear conclusion can be drawn from the different clinical reports comparing a combination of HA and SSD (HA-SSD) versus SSD alone [24]. Indeed, trials were conducted on mixed degree burns (superficial and/or deep second degree and third degree burns), with burns that differ in term of extent of the lesion and affected areas (forearm and hand, arm, trunk, lower limb) [25-28].

In this study, we used a standardized deep second degree burn rat model to allow rigorous comparison between the two treatments [a classical SSD treatment (Flammazine®) and a HA-SSD (Ialuset® PLUS)], in terms of moisture environment, epithelization rate and retraction rate.

## Materials and Methods

### Experimental protocol

Fifty-nine adult Wistar rats (250g) (Charles River Laboratories, L'Arbresle, France) received water and food *ad libitum* and were subjected to a 12/12-hour day/night cycle. Animals were anesthetized by intraperitoneal injection of xylazine (10mg/kg, Bayer Pharma, Leverkusen, Germany) and ketamine (100mg/kg, Virbac, Carros, France), shaved, and two burns were then performed successively on the back of the animal using a bronze square apparel (2x2 cm, 100°C, 15 seconds (BVG Gravure industrielle, Gergy, France)). Temperature and pressure were standardized and controlled respectively with a contact thermometer (Testo, Lenzkirch, Germany) and an electronic dynamometer (Kern and Sohn GmbH, Balingen, Germany). Burn depth was checked and protocol was standardized in preliminary experiments by histological analysis (data not shown). Buprenorphine was administered by intraperitoneal injection at the dose of 0.05 mg/kg, twice a day during 3 days. For each animal, SSD [Flammazine® (Solvay Pharma, Brussels, Belgium)] was applied on one wound and HA-SSD [Ialuset® PLUS (Laboratoires Genévrier, Antibes, France)] on the other one. To avoid bias due to the wound site, the same number of upper and lower wounds was treated in each group. Once a day, wounds were cleaned from cream residue using physiological solution (Fresenius Kabi, Sèvres, France) and disinfected with 0.2% aqueous chlorhexidine (Gilbert, Hérouville-Saint-Clair, France). Five mm of cream was then applied on the wound, and covered with sterile compress (Sylamed, Paris, France) maintained by Peha Haft (Hartmann, Heidenheim, Germany) and Urgoderm (Laboratoires Urgo, Chenove, France). Macroscopic characteristics of the wounds were blind analysed at each dressing change (i.e. infection sign, inflammatory wound, exudate presence and maceration sign). All procedures were carried out in accordance with French law and international guidelines.

### Quantitative analysis

Total wound, wound epithelization and wound retraction areas were determined using ImageJ software (US National Institutes of Health, Bethesda, MD, USA). Briefly, the total area of the wound corresponded to the area of the scar and open wound. The epithelization area was measured by subtracting the area of the open wound from the total area of the wound and expressed as a percentage of the total wound area. The retraction area was evaluated by subtracting the area from the total wound at a chosen time to the area of the initial wound at day 0 and expressed as a percentage of the initial wound area.

### Histomorphological analysis

At the end of the study, 4 animals were chosen as they had achieved complete epithelization for both treatments the same day (day 57). Scars were surgically harvested and the tissues were fixed in alcohol, formalin and acetic acid (AFA) and embedded in paraffin. Cross sections of 5 µm were stained with Hematoxylin Phloxine Saffron for histomorphological analysis. Dermis formation was scored as followed: 0 represents no re-

construction; 1: onset reconstruction; 2: emergence of blood vessels, collagen reorganization 3: blood vessels present, early annexes, collagen reorganization; 4: blood vessels and annexes present, well reorganization of collagen. Reformed and healthy epidermis and dermis thickness were measured using ImageJ software. On a same scar section, ten replicates thickness measurements of the healthy layer (dermis or epidermis) and reformed layer (dermis or epidermis) in the wound area were achieved to calculate the reformed layer/healthy layer thickness ratio.

## Statistical analysis

Changes in epithelization and retraction were compared by analysis of variance using a mixed model of ANOVA [fixed effect (treatment and time), random effect (rat)]. When a non-Gaussian distribution was present, a normalizing distribution was performed (Log transformation). Time to reach complete epithelization was compared using the Wilcoxon test for paired data followed by a survival analysis and a log-rank test. Rats with non-complete retraction were censored at the last observation and a survival analysis was performed. Comparison between treatments was made using a log-rank test. For time to reach fixed percentage of epithelization and retraction, analysis was performed using a Wilcoxon test or using a student test for paired data (according to the normality of distribution). All analysis were performed using the SAS software 9.2. (SAS Institute, Cary, NC, USA).

## Results

### Macroscopic analysis

For each animal, both burn wounds were separately treated with SSD or HA-SSD and analysed macroscopically. Wounds characteristics were scored as described in the materials and methods. No sign of infection was observed during the whole study.

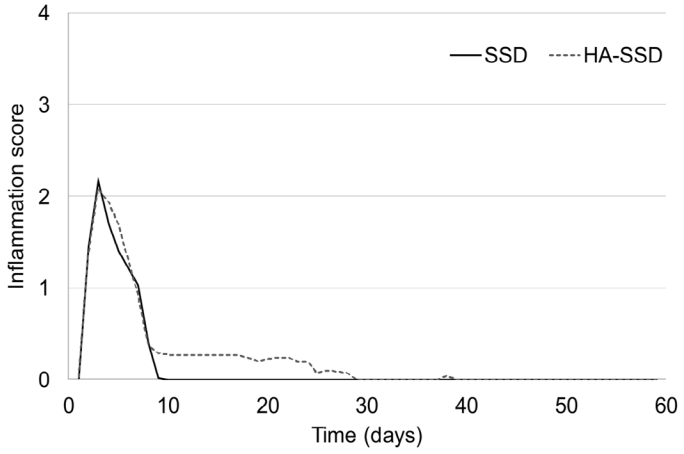


**Figure 1.** Macroscopic analysis of inflammation.

Wounds treated with SSD (black line, n=59) or HA-SSD (grey dotted line, n=59) were scored at each dressing change as follow : 0, absent; 1, slight; 2, moderate; 3, marked; 4, severe.

The inflammation score curves were identical for both treatments with a moderate inflammatory peak noticed around day 3 (2.08 ± 0.14 vs 2.17 ± 0.11 for HA-SSD vs SSD respectively) which did almost disappeared for both group at day 9 (Figure 1). Wounds treated with SSD exerted significantly more frequently exudate (81.4% ± 5.1 vs 13.6% ± 4.5 of treated wounds respectively, p<0.0001) and for a longer period (7.19 ± 0.63 vs 1.63 ± 0.26 days respectively, p<0.001) when compared to wounds treated with HA-SSD (Figure 2A and 2B). Maceration events were also significantly more recurrent for wounds treated with SSD when compared to those treated with HA-SSD (98.3% ± 1.7 versus 18.6% ± 5.1 respectively, p<0.0001) and lasted for a longer period of time (12.76 ± 0.74 days versus 2.36 ± 0.64 days respectively, p<0.001) (Figure 2C and 2D).

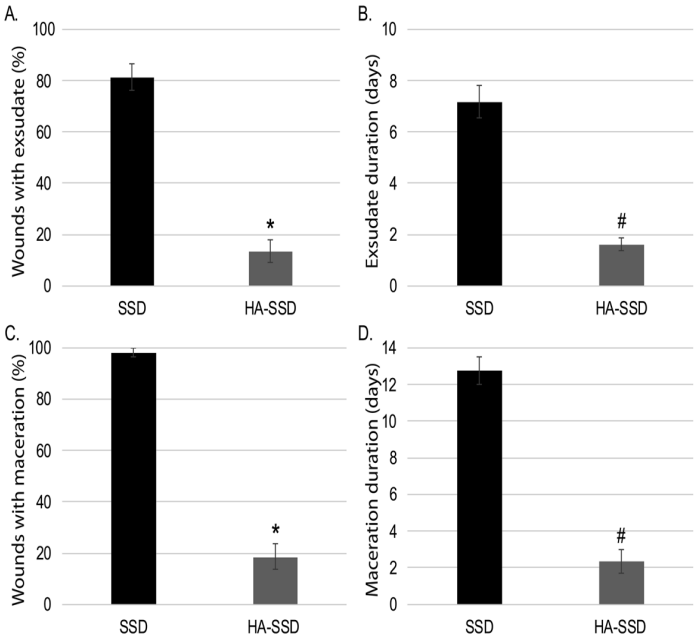


**Figure 2.** Macroscopic analysis of wound exudate and maceration

Wounds treated with SSD (black bars, n=59) or HA-SSD (grey bars, n=59) were assessed at each dressing change for wound exudate and maceration. A. Percentage of wounds exerting exudate. B. Duration of exudate presence on wounds. C. Percentage of wounds exerting maceration. D. Duration of maceration presence on wounds. Results are the mean ± SEM. *Student t-test*, * p<0.0001 and # p<0.001 considered significantly different between treatment groups.

### Quantitative analysis

Wound epithelization and wound retraction were analysed throughout the study. Epithelization rate at 28 days (mid study) was improved by 9% using HA-SSD when compared to SSD treatment (44.04% ± 1.94 versus 35.16% ± 1.86, respectively) (p<0.001). Moreover, both 25% (19.58 ± 0.48 vs 22.40 ± 0.65 days, p<0.0001) and 50% (30.91 ± 0.94 vs 35.44 ± 1.41 days, p<0.0005) epithelization rate were achieved significantly earlier when using HA-SSD versus SSD respectively (Figure 3). However, time to reach 100% epithelization was found to

be similar for HA-SSD and SSD (46.87 ± 0.80 days and 47.06 ± 0.87 days, respectively, p=0.88).

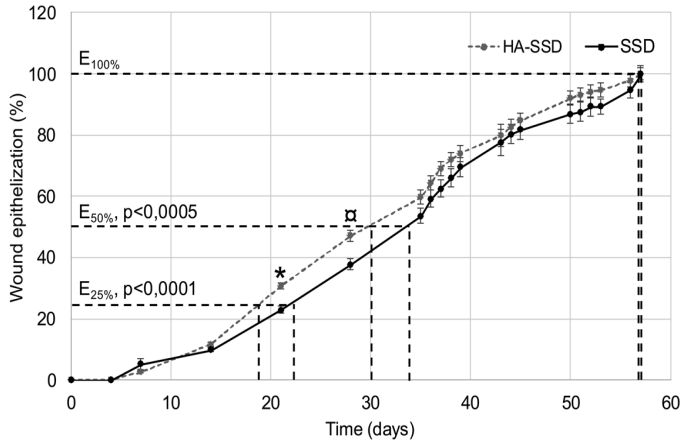


**Figure 3.** Wound epithelization kinetic

Epithelization of burn skins treated with SSD (black line) or HA-SSD (grey dotted line) were analysed using ImageJ software in term of percentage of epithelized area relative to the open wound. Projections for 25, 50 and 100% epithelization are illustrated by dotted lines. Results are the mean of 59 wounds per group. *Student t-test*, ¤ p<0.001; # p<0.0005; * p<0.0001 considered significantly different between both treatments.

The use of HA-SSD allowed a faster retraction of the wound when compared to SSD treatment. Indeed, at 28 days (mid study), wound retraction showed a 14.1% significant lead (p<0.0001) with HA-SSD (38.49% ± 1.61) when compared to SSD (24.43% ± 1.49).

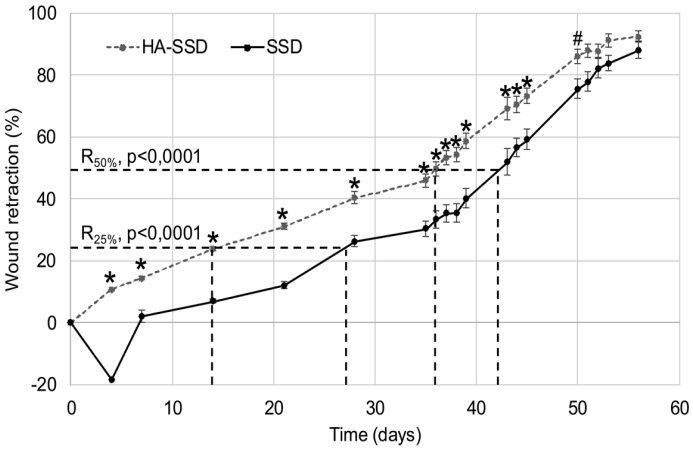


**Figure 4.** Wound retraction kinetic

Wound retraction of burn skins treated with SSD (black line) or HA-SSD (grey dotted line) were analysed as percentage of the wound area when compared to the initial wound. Projections for 25 and 50% retraction are illustrated by dotted lines. Results are the mean of 59 wounds per group. *Student t-test*, # p<0.0005; * p<0.0001 considered significantly different between both treatments.

The improvement was also significant with a gain of 15.8 days to reach 25% (13.89 ± 1.19 days vs 29.72 ± 1.28 days (p<0.0001)) and 6.6 days to reach 50% wound retraction (35.48 ± 1.08 days vs 42.09 ± 0.92 days (p<0.0001)), for HA-SSD versus SSD respectively (Figure 4). It is also important to notice that a wound enlargement phase was observed within the first days following the burn in the SSD group (characterized by a negative value for wound retraction around day 4) but not HA-SSD (Figure 4).

## Histomorphological analysis

At the end of the study (57 days post-wound), scars treated with both treatments were sampled from 4 animals, fixed in AFA and embedded in paraffin. Cross sections were stained with Hematoxylin Phloxine Saffron for histomorphological analysis. Although HA-SSD has shown slightly better results, scores for dermis formation did not emphasize differences between both treatments and remained in a remodelling phase with presence of blood vessels, the onset of appendix emergence and ongoing reorganization of collagen (scored as mentioned in the materials and methods of 2.98 ± 0.20 vs 2.74 ± 0.52 for HA-SSD vs SSD respectively, p=0.76). However, the reformed dermis thickness was significantly enhanced by HA-SSD versus SSD. Indeed, the thickness of the reformed dermis was closer to the thickness of the healthy dermis both measured on a same section with HA-SSD (geometric mean ± SEM of reformed/healthy dermis thickness ratio: 0.75 ± 0.05vs 0.43 ± 0.05 for HA-SSD vs SSD respectively, p<0.05, n=4) (Figure 5).

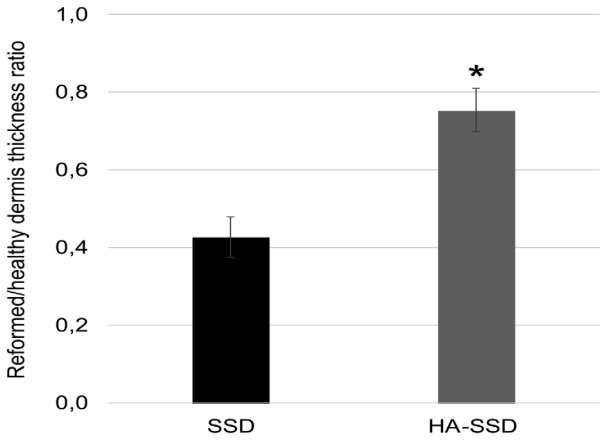


**Figure 5.** Reformed/healthy dermis thickness ratio

After 57 days, animals were euthanized and both scars treated with SSD or HA-SSD of 4 animals were sampled, fixed in AFA and embedded in paraffin. Section of 5 µm were stained with Hematoxylin Phloxine Saffron for histomorphological analysis. The mean of ten replicates for reformed and for healthy dermis thickness were measured by section using ImageJ to calculate the reformed dermis/healthy dermis thickness ratio. The higher the ratio approaches 1, the more the reformed dermis thickness is close to normal healthy dermis. Results are the geometric mean ± SEM. *Student t-test*, * p<0.05 considered significantly different between both treatments, n=4 wounds per group.

Even if no significant difference was noted, the newly epidermis was closest to the initial state in the HA-SSD group and the epidermal thickness was thinner within the SSD group (data not shown).

## Discussion

Infection remains the primary risk following severe burns injury and represents a significant source of morbidity and mortality [6]. Thus, an effective wound treatment must enhance both healing time and limits infection by maintaining an optimal moist environment and showing antimicrobial activities respectively. Effectiveness and safety of HA-SSD are well accepted by the clinical community and have been previously reported [24-28]. However, doubt persists on the real benefice versus SSD alone as recently described by Dalmedico et al [24]. Although conclusions of this review are in favour of a beneficial effect of the combination of HA and SSD, authors emphasized the need for new well-designed studies assessing the benefice versus SSD. This is mainly due to several weaknesses in the design of the existing clinical trials (i.e. wound degree burns, size, shape and body localizations) [4, 29-30]. High variability within patient age can also be a bias as ageing people present a delay in wound healing. Indeed, alteration in ageing skin (i.e. decrease of the cell numbers, the extracellular matrix components, the nerve ending and vascularity) have been shown to impair each step of wound healing (hemostasis/inflammation, proliferation and tissue remodeling) [31].

Rat and human share skin physiological and pathological characteristics. Wound contraction is the primary healing process of rats as opposed to re-epithelialization in human [32-33]. We made thus the choice to use a rat burn model as it avoids both inter and intra individual variabilities previously described and focused our experimentations on processes that are common to both species and that can be used to extrapolate to what may happen in human (wound epithelialization, dermis formation).

We have shown that, the use of a mix formulation including HA and SSD induces interesting results in terms of epithelization process when compared to SSD alone. While the time necessary to reach a complete epithelization of the wound was not significantly different between both groups, mid-study epithelization rates were largely in favour of HA-SSD (44.04% vs 35.16%, $p<0.001$). Such differences might be due to the presence of intermediary molecular weight HA (100-300kDa) within our HA-SSD formulation (Ialuset® PLUS). Indeed, HA is known to be responsible for both i) keratinocytes migration and proliferation [20,22] and ii) the generation of an optimal moist environment necessary to drive healing process [34]. Exudate and maceration events were observed for a longer period of time and almost only when using SSD. The presence of HA may then offset the water loss induced by SSD [35] leading to better epithelization, and retraction of the wound. Interestingly, a wound enlargement phase was also observed in the SSD treated group within the very first days (24-96 hours) following wound creation. As previously reported with HA alone [36], such wound enlargement phase was not observed in any animals treated with HA-SSD in our study. This may be explained by the unique properties of HA to lead fibroblast migration, proliferation and differentiation, to induce collagen synthesis, and to stimulate keratinocyte migration and proliferation which are all necessary for wound closure [20,22,37-43].

In this study, wounds were covered for extended period of times. Indeed, while this model presents many advantages in terms of reproducibility and standardization, it requires a permanent cover of wounds throughout the study to ensure that the animals cannot have access to the wounds. In patients however, once the crust is formed, wounds are left open to the air with an interruption of topical cream application. Finally HA-SSD leads to earlier epithelialization allowing for the wounds to be uncover faster compared to SSD, suggesting also a reduction of the processing cost for the patient.

## Conclusion

The gold standard in topical burn treatment remains silver sulfadiazine, a useful antimicrobial agent for burn wound treatment [44]. However, despite its beneficial effects, some adverse events on wound healing are commonly reported [13,16,44-46]. Finally, this study upholds previous results obtained in clinical trials [24-28] and supports the benefit of hyaluronic acid treatment in burn wound care. Our histomorphological analysis highlights a targeted effect on the dermal reconstruction and emphasizes a better reformed/healthy dermis thickness ratio. This is also combined with a wound epithelization and retraction gain of time with HA-SSD vs SSD. This may represent a real gain for the patient. Indeed, earlier wound closure has been demonstrated to decrease the severity of burn complications such as hypertrophic scarring, joint contractures, stiffness and to promote quicker rehabilitation [47].

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**Source of Funding**

MM Alexaline is supported by Celogos with funding from the Direction Générale de l'Armement, which belongs to the French Ministry of Defence, and from Association Nationale Recherche Technologie.

**Conflict of interest disclosure statement**

Genbiotech is a part of Laboratoires Genévrier group, the Ialuset® PLUS manufacturer.

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**Acknowledgements**

Alice Antier has participated to the histological analysis under the supervision of MM Alexaline.

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