The Effectiveness of the Haemodialysate Solcoseryl® for Second-Intention Wound Healing in Horses and Ponies

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With 6 figures

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Summary

Second-intention healing of limb wounds in horses is often problematic. Solcoseryl® is a protein-free, standardized dialysate/ultrafiltrate (HD) derived from calf blood, which has been shown to improve healing in both animals and humans. The efficacy of HD in the healing of deep wounds in horses and ponies was investigated. Deep wounds of 20 by 35 mm were created on both metatarsi (skin, subcutis, periosteum) and on both femoral biceps muscles (skin, subcutis, muscle) of five horses and five ponies. The wounds on one side were treated with HD, four times a week during the period that the wounds were bandaged and once daily thereafter. The wounds on the other side were left untreated. In the first 4 weeks of the healing period HD stimulated healing but inhibited healing thereafter. This pattern was significant for all wound groups ($P \geq 0.001$). Because of this change in effect, the overall effect on wound healing over the entire period was not significant ($P = 0.77$). HD stimulated healing initially by provoking a greater initial inflammatory response, faster contraction and faster formation of granulation tissue. Subsequently, HD inhibited healing because it significantly delayed epithelialization and caused protracted inflammation. The effects of HD were most pronounced in the horses. Because this study distinguished between contraction and epithelialization, it could be shown that HD stimulated contraction but inhibited epithelialization. Therefore, HD is useful in horses for the treatment of deep wounds during the initial phase of healing by second intention, i.e. during the first weeks when wound contraction can be expected. Treatment should be ceased when epithelialization becomes predominant.

Introduction

Traumatic wounds in horses often heal by second intention because many wounds cannot be sutured for various reasons. Moreover, dehiscence often occurs after primary closure. Wounds healing by second-intention healing are prone to complications. Persistently swollen limbs, lameness and ugly scars frequently result. Therefore, there is a great demand in equine practice for products that speed up second-intention wound healing, thus improving the final cosmetic appearance and enhancing the chances for a return to full athletic performance.

Solcoseryl® (Solco Basle Ltd, Birsfelden, Switzerland), referred to as haemodialysate (HD) in this article, is a protein-free, low molecular weight fraction derived from haemolysed calf blood by dialysis/ultrafiltration. HD improves second-intention wound healing in several species by interacting with different steps of the wound healing cascade. HD stimulates the healing of ulcers and burns in humans (Knudsen et al., 1982; Marichy and Eyraud, 1984; Biland et al., 1985; Rossano et al., 1990; Rossano et al., 1993) and also the healing of experimental wounds in other species, such as rats (Isler et al., 1991a; Isler et al., 1991b), pigs (Hoekstra, personal communication) and guinea-pigs (Laaf, 1993). In vitro, HD stimulates cell metabolism, growth and migration (Riede et al., 1975; Niinikoski and Renvall, 1979; Fraefel et al., 1985; Dri
et al., 1989; Fabbro et al., 1992; Schreier et al., 1993; Spessoto et al., 1993; Miltenburger et al., 1994).

Although HD has been shown to improve the healing of superficial wounds in horses (Jöchle and Hamm, 1983), its effect on traumatic wounds deeper than full-thickness skin wounds, which are far more common in veterinary practice, is not known. Moreover, little is known about its mechanism of action.

The mechanisms of second-intention healing have been proved to be different for horses and ponies (Wilmink et al., 1999a; Wilmink et al., 1999b), and therefore the effect of treatment could also be different in these two groups.

The purpose of this study was to evaluate the effectiveness of HD on second-intention healing in horses and ponies. To this end, standardized deep excisional wounds were made on both metatarsi and both femoral biceps muscles of five horses and five ponies. The wounds on one side of each animal were treated with HD and the wounds on the other side were left untreated. The healing process was evaluated both macroscopically and histologically.

**Materials and Methods**

This study forms part of a larger study of wound healing in equines (Wilmink et al., 1999a,b). It was approved by the Ethics Committee of the University, in compliance with the Dutch Act on Animal Experiments. Full details of the materials and methods are given elsewhere (Wilmink et al., 1999a,b).

**Horses and experimental set-up**

Five Shetland ponies (mean body weight 136 kg, range 121–154 kg) and five Dutch Warmblood horses (mean body weight 467 kg, range 376–529 kg), all 2-year-old geldings, were used. Rectangular tattoos (30 by 45 mm) were made at the future wound sites. Four weeks later under general anaesthesia, a standard experimental wound was created at predefined sites on both metatarsi and both femoral biceps muscles of each animal by cutting along a template of 20×35 mm. On the limbs, skin, subcutis and periosteum were surgically removed and on the buttocks, skin, subcutis and muscle were removed to a depth of 18 mm. Superficial necrosis was induced by freezing the wounds for 6 s with a brass block cooled to –80°C with liquid nitrogen.

The wounds at one side of the animal were treated with HD, and those at the other side were left untreated. Sides were randomly assigned. Wound management was the same for all wounds. All wounds were covered with non-pressure bandages for the first 3 weeks. The bandages were changed four times a week, and at the same time a thin (1 mm) layer of HD gel (8 mg/g) was applied to the HD-treated wounds. Thereafter, the wounds were left uncovered and those on the treated side were treated with a thin (1 mm) layer of HD ointment (2 mg/g) every day. Granulation tissue protruding more than 3 mm was trimmed to the level of surrounding skin at week 4. Treatment was discontinued at week 9. Throughout the study all animals had a good appetite, they did not become lame, nor did they show signs of discomfort.

**Wound evaluation**

The wounds were evaluated over a 12-week period. For this study the results are given only for the first 9 weeks because HD treatment was ended after this period. Photographs of all wounds were taken weekly. Rulers were held vertically and horizontally close to the wound as a reference, to allow for correction of wound areas. The wound and tattoo areas were measured on the photographs using plane geometry. Each area was corrected for any possible variation in focus distance, using the rulers. Additionally, the areas of the metatarsal wounds were corrected for the curvature of the metatarsi.

Wound contraction and epithelialization were calculated. Wound contraction was expressed as a percentage of the wound area on Day $D_x$ defined as the day of the maximal wound size. This maximal wound size was reached after the initial enlargement that followed the creation of the wounds. The area of newly formed epithelium was also calculated. The following formulae were used (Fig. 1): 

$$\text{% contraction at } D_x = \left(\frac{\text{tat}_D - \text{tat}_D_0}{\text{wnd}_D} \right) \times 100; \text{ and epithelialized area at } D_x = \text{tat}_D - (\text{tat}_D_0 - \text{wnd}_D) - \text{wnd}_D;$$

where tat is the tattoo area, wnd is the wound area and D is day.

In these formulae it is assumed that the area of the rim of skin between the tattoos and the original wound margin (i.e. tat$D_0$–wnd$D_0$) is a constant value throughout the healing process.

The swelling of the limbs was measured with a tape measure at the proximal and distal borders of
the tattoos and the mean value was calculated. Radiographs of the limbs were taken at weeks 3 and 6 to detect sequestration and to evaluate the extent, density and activity of periosteal new bone formation.

**Biopsy procedure**

Each week surgical biopsies were taken under general anaesthesia from all wounds of one horse and one pony. A thin slice of tissue was excised from the wound margin to the centre of the wound over the complete depth. The biopsies included a small portion of the original epithelium to facilitate orientation during microscopic evaluation. Each week the biopsies were taken from another horse and pony, starting again with horse 1 and pony 1 at week 6. Sampling was discontinued when the wound had closed to such an extent that the biopsies would consist of more than half the remaining wound area. This happened in week 6 for some wounds. For this reason histological results are only presented for the first 6 weeks.

**Histological processing and evaluation**

The biopsies were embedded in paraffin wax and 5-μm sections were cut. These were stained with haematoxylin and eosin (H&E), with Giemsa for cell counts, with phosphotungstic acid haematoxylin (PTAH) for fibrin, with van Gieson Elastica (GEL) for collagen and elastin, and with Gram’s stain for bacteria. Monoclonal antibodies (Biogenic Laboratories, San Remo, CA, USA) were used to detect smooth muscle actin (SMA) employing a immunohistochemical ABC method. Mitotic activity was visualized with monoclonal antibodies (Immunotch, Marseille, France) against the nuclear Ki 67 antigen which is expressed in DNA-synthesizing cells (MIB).

Each section was divided into a number of horizontal zones with a depth equal to the width of one field of vision at a magnification of × 200 (approx. 1 mm). In each zone, at sites adjacent to the edge of the newly formed epithelium and in the centre of the wounds the following variables were evaluated quantitatively or semiquantitatively.

- Cells were counted at a magnification of × 400 (Zeiss M100 microscope with calibrated ocular piece). Giemsa staining was used for polymorphonuclear leucocytes (PMNs), mononuclear cells and fibroblasts, and MIB staining was used for cells synthesizing DNA.
- Smooth muscle actin content and the organization of myofibroblasts were scored in SMA staining, as was collagen formation and crimp after GEL, fibrin after PTAH and bacteria after the Gram staining.

**Statistical analysis**

The results were statistically analysed using a multivariate repeated measurement analysis. Significance was set at $P < 0.05$.

**Results**

HD had a significant effect on second-intention healing of deep experimental wounds. It led to a sinusoidal pattern in the healing curve when compared to the untreated wounds (Fig. 2), with stimulation of healing in the first 4 weeks, but inhibition afterwards. This pattern was seen in all wound groups (horses, ponies, metatarsal and muscle wounds). The mean area of all
wounds treated with HD was as much as 7% smaller than that of the untreated wounds in the first 4 weeks. However, from then on the healing of the treated wounds slowed down, and the mean area of these wounds became as much as 36% greater than that of the untreated wounds (Fig. 2). The mean maximal stimulation and inhibition of healing were of the same order, in both cases 9% of the initial wound area on Day 0. The effect of treatment with HD was most obvious on the metatarsal wounds of the horses. In these wounds the difference between untreated and treated wounds was significant at weeks 3 and 4, the wound area of the treated wounds being 16% smaller than that of the untreated wounds at week 4 (Fig. 2). However, the wound area was finally 27% greater than that of the untreated wounds at week 8. Expressed as a percentage of the initial wound area on Day 0, stimulation was 28% and inhibition was 19% in the metatarsal wounds of the horses.

HD affected wound healing in four ways. First, it tended to stimulate wound contraction, particularly in the metatarsal wounds of the horses. Histologically, the myofibroblasts, which cause contraction, became regularly organized sooner in the treated than in the untreated wounds (Fig. 3). In the ponies, the myofibroblasts were already organized in an early stage of healing, and HD did not affect this further.

Secondly, HD significantly inhibited epithelialization in the wounds of the horses from week 5 onwards. From then on, the epithelialized area of the treated metatarsal wounds was on average 16% smaller than that of the untreated metatarsal wounds. In the treated muscle wounds, this difference was on average 22% (Fig. 4). Epithelialization of treated and untreated wounds in the ponies did not differ. Histologically, the number of epithelial cells that synthesized DNA was slightly lower in the treated than in the untreated wounds.

Thirdly, HD stimulated the inflammatory response. It stimulated exudation, and the exudate became purulent sooner in the treated wounds. Moreover, the surface of the granulation tissue remained purulent longer in the treated than in the untreated wounds. Histologically, there were more PMNs in the granulation tissue of the HD-treated wounds during the entire observation period (Fig. 5). This difference in PMN number was greatest for the horses’ wounds. The number of PMNs in the untreated metatarsal wounds of the horses was initially particularly low compared with the untreated muscle wounds and the untreated pony wounds. However, after treatment with HD, PMN numbers increased markedly: in week 2 there were more than twice as many PMNs and in week 3 more than three times as many PMNs in the treated than
in the untreated wounds (Figs 5, 6a,b). In contrast, the number of PMNs in the untreated wounds of the ponies was already high during the first 3 weeks of healing and HD did not increase the number further. Although the numbers of PMNs decreased in all wounds after weeks 2 and 3, there remained almost twice as many PMNs in treated than in untreated wounds (Fig. 5). Also, slightly more mononuclear leucocytes were seen in the treated wounds and fibrin depositions disappeared earlier.

Fourthly, HD stimulated the formation of granulation tissue slightly but persistently. In most wounds some excessive granulation tissue was observed from week 2 onwards, but this often reduced spontaneously. However, in the HD-treated wounds some excessive granulation tissue (less than 3 mm) remained during the rest of the healing period. At week 4, both metatarsal
wounds of three horses had to be trimmed. Histologically, on average 2.5% more cells showed mitotic activity in the treated than in untreated wounds during the observation period. This increased activity was most obvious in the metatarsal wounds of the ponies.

There was no difference in limb swelling and periosteal new bone formation between treated and untreated wounds. Nor was there a difference in the mean number of fibroblasts and collagen formation. Bacteria were only found in the metatarsal wounds and the treated muscle wound of horse 1 at week 1. None of the other wounds contained bacteria throughout the entire observation period.

**Discussion**

A significant effect of HD treatment on the pattern of wound healing existed in all wound groups (horses, ponies, metatarsal and muscle wounds), and consisted of stimulated healing during the first 4 weeks but inhibited healing thereafter. This effect can be explained by a stimulation of inflammation, granulation tissue formation and wound contraction. These processes are essential in the early phase of wound healing. However, the delay of epithelialization and the persistence of inflammation lead to inhibition of healing in the later phase. This change in effect resulted in a non-significant net effect of HD over the whole 9-week period.

The effect of HD treatment also varied between the wound groups. This is probably because the mechanisms of second-intention healing differ not only between various species, but also within the same species. Ponies show a strong but short inflammatory response and a fast and pronounced wound contraction, whereas horses show a weak but protracted inflammatory response, less contraction and consequently more epithelialization (Wilmink et al., 1999a,b). In the literature, HD appears to be most effective when the wound healing process is impaired, or when in *vitro*, culture conditions are suboptimal (Knudsen et al., 1982; Marichy and Eyraud, 1984; Biland et al., 1985; Rossano et al., 1990; Rossano et al., 1993; Spessoto et al., 1993; Miltenburger et al., 1994). In this study, HD also affected the impaired phases of healing more than it affected the other phases. Therefore, the beneficial effect of HD was most obvious on the metatarsal wounds of the horses, which are notorious for problematic wound healing. HD had little effect on the fast healing wounds of ponies.

HD has been shown to stimulate wound healing in many species, such as guinea-pigs (Laaf, 1993), rats (Fraefel et al., 1985; Isler et al., 1991a; Isler et al., 1991b), and also horses (Jöchle and Hamm, 1983; Liebich et al., 1988). In none of these studies was a clear distinction
made between closure of wounds by wound contraction or epithelialization, and inhibition of epithelialization was not observed. Because the present study distinguished between contraction and epithelialization, it could be shown that HD stimulated contraction and inhibited epi-
There is no doubt that, in the older literature, enhancement of healing was often incorrectly attributed to a stimulation of epithelialization. For example Jöchle and Hamm (1983) showed, that in horses, HD treatment gave rise to smaller scars without a change in hair colour. This can only have been the result of contraction because epithelialization results in scar formation. In the microscopy section of the same study (Liebich et al., 1988), epithelialization was indeed slightly slower, and collagen formation and arrangement were stimulated. The latter indicates that wound contraction is stimulated, since this depends upon the organization of myofibroblasts (Wilmink et al., 1999b), and the organization of myofibroblasts and collagen arrangement occur simultaneously (Stopak and Harris, 1982). Jöchle and Hamm did not find an inhibition of healing in the later phases of wound healing. This may be due to the shorter evaluation period (34 days) and the fact that they investigated superficial wounds. This HD-induced inhibition of epithelialization was most obvious in the horse wounds, because pony wounds close mainly by wound contraction (Wilmink et al., 1999a). Epithelialization could not be determined accurately in the first 3 weeks due to the presence of exuberant granulation tissue.

HD stimulated the inflammatory response throughout the treatment period. An adequate inflammatory response is a prerequisite for rapid wound healing (Silver, 1982), and the intensity of the initial inflammatory response is positively related to the speed of wound healing (Chvapil et al., 1991). Indeed, differences in initial inflammatory response between horses and ponies were found to correspond with the differences in speed of healing (Wilmink et al., 1999a,b). This finding suggests that the initial inflammatory response may contribute to the observed differences in second-intention healing between horses and ponies. The stimulation of the inflammatory response by HD treatment was most pronounced in the metatarsal wounds of the horses and may have resulted in the faster healing of the treated wounds. This is consistent with earlier findings for the rat (Isler et al., 1991a; Isler et al., 1991b) and the horse (Liebich et al., 1988), with the exception that in these studies the inflammatory response decreased earlier in the treated than in the untreated wounds, whereas it remained stimulated throughout the healing period in our study. Though the initial inflammatory response is essential for adequate wound healing, chronic inflammation with persistent involvement of PMNs may lead to cell destruction and an altered composition of the extracellular matrix, with the subsequent failure of epithelialization (Cotran et al., 1994; Knottenbelt, 1997). Therefore, as stated earlier, the persistent inflammation in the treated wounds may underlie the inhibition of wound healing seen after the first 4 weeks.

HD stimulated the formation of granulation tissue and DNA synthesis, which agrees with the increased proliferation and migration of fibroblasts seen in vivo and in vitro in other studies (Riede et al., 1975; Niinikoski and Renvall, 1979; Fraefel et al., 1984; Laaf, 1993; Schreier et al., 1993; Miltenburger et al., 1994). The mitotic activity of fibroblasts was stimulated during the entire healing period. Although such activity initially enhances healing, when the wound has to be filled up by granulation tissue, it will ultimately lead to an excess of granulation tissue and therefore to inhibition of epithelial mitosis (Wilmink et al., 1999b).

The mechanism of action of HD is still speculative. HD is protein-free, but it contains oligopeptides as well as autolytic fragments of higher molecular weight proteins. It does not contain growth factors, with the exception of minute amounts of EGF, which are insufficient to explain any effect on healing (Konturek et al., 1991). Nevertheless, HD influences fibroblast migration and proliferation in vitro in the same way as basal fibroblast growth factor, platelet-derived growth factor and transforming growth factor-β (Schreier et al., 1993), and thus has a growth factor-like action, possibly by stimulating growth factor receptors. It also promotes the differentiation of human monocytes into macrophages under suboptimal culture conditions (Spessoto et al., 1993). Macrophages play a key role in inflammation and particularly in wound healing and tissue remodelling because they produce numerous cytokines and growth factors which affect fibroblast proliferation, collagen synthesis, endothelial cell migration and angiogenesis. Of the growth factors, transforming growth factor-β is of special interest because it stimulates the proliferation of fibroblasts and collagen synthesis (Sporn et al., 1986). It also increases the contraction of fibroblasts (Tingström et al., 1992) and inhibits the proliferation of epithelial cells (Sporn et al., 1986).
In conclusion, HD initially stimulates second-intention wound healing in horses. The increased inflammatory response enhances the demarcation of non-viable tissues. Subsequently, the migration and proliferation of fibroblasts is stimulated, including their differentiation into myofibroblasts, thus promoting wound contraction. This is achieved either directly by the growth factor-like action of HD or indirectly as a result of the increased number of macrophages. After this initial period, HD has a negative effect on wound healing because epithelialization is inhibited, probably by a direct action of HD or as a result of the protracted inflammation. The observation that the effect of HD is most pronounced in the metatarsal wounds of the horses, where wound healing is known to be suboptimal, is consistent with a greater efficacy of the drug under adverse conditions. In practice, HD can be used in the initial phase of second-intention wound healing in the horse when inflammation and granulation are needed and contraction can be expected, but is contraindicated in the later phases when epithelialization becomes predominant. In addition, this study again emphasizes the importance of further research in equine wound healing, and it indicates that the inflammatory reaction may be very important. A better understanding of the pathogenetic pathways and a better knowledge of the cytokines and growth factors involved may help us to elucidate the exact mechanisms and may contribute to the development of therapies directed towards specific inadequacies in equine healing.

References


