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Original Article

HDFx: a novel biologic immunomodulator accelerates wound healing and is suggestive of unique regenerative powers: potential implications for the warfighter and disaster victims

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Abstract: Recently, we reported on the discovery of a new, conserved biologic protein (35-40 KDa), termed HDFx, that protects rats, guinea-pigs, mice, and rabbits against lethal hemorrhage, endotoxins, intestinal ischemic-shock, and traumatic injuries. It was found to stimulate several arms of the immune system. The present report demonstrates, for the first time, that HDFx accelerates wound healing in two different models (excision wound model; and incision wound model) in rats. The results shown, herein, indicate that HDFx produces greater rates of wound contraction, greater tensile strength, and more rapid healing than controls. Our new data also show that this biologic increases hydroxyproline content of granulation tissue coupled with a reduction in superoxide dismutase (SOD). In addition, we show that HDFx increases the levels of serum ascorbic acid and stimulates the mononuclear cells of the reticuloendothelial system (RES). Overall, these data suggest that HDFx may possess unique regenerative powers. We, thus, believe that HDFx can be of great potential use in diverse types of wounds which, otherwise, could result in difficult to treat infections and thus prevent sepsis and loss of body parts from amputations.

Keywords: Wound contraction, tensile strength, wound healing, hydroxyproline, SOD, reticuloendothelial system

Introduction

Diverse types of wounds are inflicted on the human body each year through infections, accidents, surgery, violence, diabetes, vascular disorders, cancers, debilitation and war. Often, many of these wounds become chronic. It is often quite difficult to treat many chronic wounds which can result in scars and disfigurement. These chronic wounds can become painful and dangerous, often resulting in sepsis and antibiotic-resistant microorganisms, gangrene and amputation. The debilitated chronically-ill, surgical patient is at high-risk and particularly susceptible to slow-healing wounds.

At present, the U.S. armed forces and the civilian population is at considerable risk from vesicant agents. Usually, treatment consists of wound debridement, followed by skin grafting and the application of various types of wound dressings. However, these regimens are often limited in the benefits they offer. Much of the problem resides in the slowness of wound healing which results in considerable suffering for the patients.

The continued use of improvised explosive devices (IEDs) in Iraq and Afghanistan has resulted in marked increases in untoward injuries from blast trauma producing numerous severe challenges in overcoming severe head, facial, limb, and hand injuries that often take not just months, but years of continuous surgeries and treatments of various types which often result in major lifelong impairments. Much of this is due

to delays in the healing process of wounds.

Currently, methods used to treat wounds comprise debridement, irrigation, use of antiseptic agents, antibiotics, corticosteroid therapies, and tissue grafts.

Recently, our group has reported the discovery of a naturally-occurring biologic in several species of rodents and rabbits which we have termed HDFx that stimulates several arms of the innate immune system and leads to protection against lethal hemorrhage, trauma, bowel ischemic –shock, endotoxins, sarcoma-180 and combined injuries when administered as a prophylactic treatment [1]. We have also found that HDFx when administered to animals post-hemorrhage and post- intestinal ischemic shock leads to increased survival , elevation of arterial blood pressure, near-normal phagocytic function of macrophages, amelioration of deterioration of the immune system, and near-normal microvascular function [2]. In the course of our experiments, we noticed that the injured animals appeared to heal faster than their control littermates. These observations suggested that HDFx might possess unique regenerative attributes.

The present studies were undertaken to determine whether prophylactic treatment, of animals subjected to wounding, would result in decreased time of wound healing, better healing of wounds (e.g., earlier wound closure; increased tensile strength of the skin), significant increases in hydroxyproline and collagen content, and significant decreases in malondialdehyde content in the wounded animals. In addition, phagocytic activity of mononuclear cells of the RES were measured as these factors are known to be upregulated in wound healing [3, 4].

Animals and methods

Animals, anesthesia, precautions taken and survival studies

Young, adult male inbred Wistar strain rats (165 -200g) were used for all studies. All animals were anesthetized prior to surgery and wounding with pentobarbital sodium (Nembutal, 5 mg/100g i.m.). To reduce genetic variability as a factor [5, 6], littermates from our breeding colony were used throughout these studies [1, 2]. All animals were at least 70 days old and, thus, sexually mature [1]. Only male animals were included as the sex of animals has been repeatedly demonstrated by a number of workers to influence the outcome of trauma-stress studies and the responses of macrophages and other cellular elements of the immune system to pathophysiologic stimuli [5, 7-9]. All animals were given sterilized distilled water to drink and Purina rat chow pellets ad libitum [1]. Aseptic techniques were utilized throughout the studies [1, 2]. Each study group (controls and experimental groups) were comprised of 10-16 rats. Each animal, prior to surgery or animal model procedure, was placed on a temperature – controlled table and maintained under temperature -controlled conditions [1, 2]. A femoral artery and vein were cannulated in each animal for monitoring of arterial blood pressure and i.v. infusion of either saline, additional anesthesia, or drugs. All animals were carefully monitored for survival for at least 32 days. Buprenorphine (50 ug/kg) was given every 8 hr during the post-wounding period, two-times/day on day 2, and two –times/day on day 3. If any animal demonstrated any excess pain (despite buprenorphine administration) or other untoward effects, it was sacrificed with a lethal dose of pentobarbital sodium (i.e., 100mg/100g). This investigation conformed to the *Guide for the Care and Use of Laboratory Animals* published by the U.S. National Institutes of Health (NIH Publication No. 85-23, revised in 1996) and was approved by The *Animal Use and Care Committee at SUNY Downstate Medical Center.*

Excision wound model

A full-thickness excision wound (circular area approximately 500 mm^2) and 2-mm deep was undertaken after each rat was shaved on its dorsal surface one day prior to the wounding procedure. The wounded area was left undressed and exposed to its environment. The degree of wound closure was studied and measured by tracing the raw wound using a transparent paper and a permanent marker on every 4^{th} day for 16 days and tracing the wound area on millimeter scale graph paper [10]. As demonstrated previously, the epithelization period was calculated as the number of days required for the dead tissue remaining to fall off without any demonstration of the residual raw wound [11]. On the 10^{th} day, the scab was removed and utilized for estimation of hydroxyproline content

[12]. Blood samples were taken and plasma separated for estimation of malondialdehyde (MDA) [13] and ascorbic acid [14].

Collection of granulation tissue for hydroxyproline and superoxide oxide (SOD) estimation

We collected granulation tissues from the wounded areas (in saline controls and HDFx-treated animals) on the 10th day. They were washed in ice-cold normal, sterile saline solution and lyophilized for L-hydroxyproline estimation [12]. The method of Misra and Fridovich was utilized to estimate SOD activity [15].

Wound contraction

Wound contraction is a useful measurement and is useful in order to assess wound healing. This was assessed as % wound contraction= healed area X100/ total wound area. The wound area was measured at 4-day intervals by tracing the wound margin using transparent paper. The total healed area was calculated by subtracting its area from the original wound area [11].

Incision wound model

After the animals were anesthetized (as above) ,and the skin shaved on the dorsal surface, we made a single incision of 5 cm in length and 2 mm in depth with a sterilized scalpel in both control and HDFx-treated rats. The cut wound was then held together and sutured with surgical silk at, approximately, 0.5 mm intervals. The contiguous thread was tightened until closure. After the wounds were thoroughly healed, the animals were then anesthetized again (as above) and the sutures were removed on the 8th day in both controls and HDFx-treated rats. On day 10, the tensile strength (wt in gm required to break the wound open) was measured in anesthetized animals using a tensiometer, similar to that previously reported [16]. The tensile strength was considered to be the amount of wt needed to break the wound. All animals were then sacrificed using a lethal dose of pentobarbital sodium (100 mg.kg).

RES phagocytic function

The procedure in these experiments essentially consisted of determining RES phagocytic indices (K- values) [17, 18] in the separate and different groups of experimental animals identical to the models above. The phagocytic indices were determine at 3, 24, and 96 hr after wounding both in the control and HDFx-treated groups by measuring the rate of clearance of colloidal carbon(4 mg in calf skin gelatin/100gm body wt) [17, 18]. Precisely timed blood samples were obtained at 2, 4, 8, 12 and 15 min after the i.v. colloidal carbon injection, hemolyzed in 0.1 % sodium carbonate and the carbon concentration measured photometrically at 675 mu [17, 18]. The colloidal carbon utilized in these studies was Pelikan C11/1431 (Gunther-Wagner, Hanover, Germany). Phagocytic indices were calculated:

$K = \log_{10}C_1 - \log_{10}C_2/t_1 - t_2$

Where K is the phagocytic index and C_1 and C_2 are the colloidal carbon concentrations in mg/100 ml of blood at times t_1 and t_2 [17, 18].

Isolation, preparation and standardization of HDFx for systemic administration

Briefly, heparinized blood was obtained from control, naïve rats stimulated to produce HDFx in adaptation to whole-body LD 80- trauma, as shown previously [1, 2]. Precautions were taken for blood collections as described [1, 2]. All blood collection tubes and glassware were tested for endotoxins using a Limulus Amebocyte assay as described [1, 2].We set one unit of purified HDFx to be equivalent to raising the RES phagocytic index (K-value) 100% in naïve rats [1, 2]. Two units of HDFx raised the RES phagocytic index over 200% in naïve rats [1]. This latter dose was administered to the experimental rats on day -1, and days 2-4 via i.v. injections (tail vein).

Statistical analyses

Where appropriate, means and means +/- S.E. were calculated. Differences between means were assessed for statistical significance by Student's t-test and ANOVA, followed by a Newman-Keuls test. A P-value < 0.05 was considered significant.

Results

Wound contraction

The data presented in **Table 1** demonstrate that the wound contractions of the HDFX-treated

Table 1. Effects of HDFx pretreatment on wound healing and epithelialization in the excision wound model in rats

Group	% Wound contraction				Period of epithelialization (days)
	4th day	8th day	12th day	16th day	
Wounded Controls	4.95 +/-0.75	23.7 +/-0.15	53.77 +/- 1.1	72.4 +/- 0.4	30
HDFx	!6.4 +/- 1.12*	50.6 +/- 0.56*	82.4 +/- 0.8*	99.2 +/- 0.26*	18

Values are means +/- S.E.M. *Values significantly different from controls (P< 0.001).

Table 2. Effects of HDFx on hydroxyproline(HP) content of granulation tissue and the serum levels of malondialdehyde(MDA) and ascorbic acid(AA)

Group	HP, mg/g tissue	MDA, nmoles/mg protein	AA, g/dl
Wounded control	8.12 +/- 0.26	0.792 +/- 0.042	2.24 +/- 0.18
HDFx	20.24 +/- 0.82*	0.224 +/- 0.018*	6.46 +/- 0.38*

Values are means +/- S.E.M. *Values are significantly different from controls(P<0.001).

groups were significantly higher at all times after wounding when compared to the untreated controls (P<0.001, ANOVA). It is of considerable interest to note that the period of epithelization was vastly accelerated in the HDFx-treated group (P<0.001).On day 18 , the wounds treated with HDFx were completely healed as compared to the controls which took 30 days to heal.

Hydroxyproline content, MDA, and ascorbic acid

It is clear from the results in Table 2 that the hydroxyproline content of the granulation tissue of the HDFx-treated animals was more than 100% higher than that in the untreated controls. Likewise, the serum level of ascorbic acid was more than 75% higher than the untreated controls (**Table 2**). Interestingly, the serum level of MDA was reduced more than 75%.

Tensile strength in excision wound model

Measurement of tensile strength in the excision wound model indicated that the HDFx-treated animals exhibited almost a 100% increase in tensile strength (P<0.001) (**Table 3**).

Superoxide dismutase (SOD) activity in granulation tissue

The results in **Table 4** demonstrate that the SOD activity in the granulation tissue was reduced approximately 80% compared to the untreated controls at day 10.

Table 3. Effects of HDFx on tensile strength in the incision wound model

Group	Tensile strength (g)
Wounded controls	292 +/- 4.22
HDFx	542 +/- 21.8*

Values are means +/- S.E.M. *Values significantly different from controls (P<0.001).

Table 4. Effects of HDFx on superoxide dismutase (SOD) in granulation tissue of wounded rats 10 days after injury

Group	SOD, mg/100 mg tissue
Wounded controls	18.2 +/- 1.44
HDFx	2.82 +/- 0.18*

Values are means +/- S.E.M. *Mean value significantly different from controls (P<0.001).

RES phagocytic function after wounding

The data presented in **Table 5** indicate that early after wounding in the controls, the RES phagocytic index is depressed significantly (more than 70%) compared to naïve unwounded animals, whereas the HDFx-treated animals exhibit only a 15% decrease in phagocytic capability (P<0.001, ANOVA). On day -4 , while the untreated wounded controls still exhibit a significant depression in phagocytic capability, the HDFx-treated animals ,surprisingly, demonstrate a stimulation in RES phagocytic capability which continues to be manifest even

Table 5. Effects of HDFx on RES phagocytic indices after injury in the excision wound model in rats

Group	Time after wounding, hr		
	3	24	96
Naïve controls	0.045 +/- 0.002	0.043 +/- 0.003	0.048 +/- 0.004
Wounded controls	0.008 +/- 0.002*	0.022 +/- 0.004*	0.032 +/- 0.004*
HDFx	0.038 +/- 0.004**	0.076 +/- 0.004**	0.062 +/- 0.006**

Values are means +/- S.E.M. *Mean values significantly different from controls (P<0.01). **Mean values significantly different from all other paired –timed values (P<0.001, ANOVA).

at 96 hr after wounding unlike the untreated controls (**Table 4**).

Discussion

Overall, we believe that, collectively, our experiments demonstrate that the discovery of HDFx [1, 2] represents a new biologic tool in allowing severe wounds to be healed faster and better than a variety of known drugs and therapeutic interventions. Our new experiments clearly indicate that HDFx possesses a tempting array of characteristics known to be important in the healing of wounds: increased epithelization; increased wound contraction; increased tensile strength; increased and more rapid production of hydroxyproline in the granulation tissue coupled to increased serum levels of ascorbic acid concomitant with marked reductions in the production of free radicals (exemplified by reduction in serum MDA levels and lowered SOD tissue levels).

Normally, after wounding injuries revascularization of the wounded beds begin to take place followed by a redevelopment of the extracellular matrix through cell proliferation and the production of granulation tissue [19-21]. Contraction of the wound(s) is a part of the proliferative phase of wound healing [19-21]. Increased wound contraction in the HDFx-treated groups may be due to enhanced activities of fibroblasts and increased activity (and elimination) of bacteria by phagocytic cells of the RES-monocyte systems [1, 5]. Our present results indicate that the HDFx-treated animals exhibit higher than normal RES phagocytic capabilities which probably play a major role in the accelerated healing of the HDFx-treated rats. In this context, we have shown recently that stimulation of the RES-monocyte system by HDFx plays a major role in protecting animals against the lethal effects of gram-negative bacteria, intestinal ischemic shock, hemorrhage, and lethal body trauma [1, 2]. The slower rates of wound healing in the control group may be attributed to a slowness in the clearance of bacteria from the wounds. Our present findings would be consistent with this hypothesis.

During the wound healing process, fibroblasts and epithelial cells normally migrate to the wound sites [19-21]. The increased collagen content found in the granulation tissue of the HDFx-treated group is probably a result of the stimulation of fibroblast and epithelial movements by various attributes of HDFx, particularly as we have demonstrated, previously, that HDFx has the power to increase numbers and sizes of various cells in the immune system, as a consequence, in part, of production of cytokines, chemokines, and growth factors [1, 2]. Our present results clearly indicate that HDFx –treatment results in increased levels of ascorbic acid, an important co-factor for the synthesis of collagen. The observed finding of increased hydroxyproline in the HDFx-treated animals suggests there is increased turnover of collagen. Our findings of increased tensile strength in the HDFx-treated rats serves to reinforce our hypothesis that the increased turnover of collagen is, indeed, occurring in these animals since it is known that the tensile strength of a wound is a reflection of the rate of collagen biosynthesis where one finds a covalent binding of collagen fibrils via intra-molecular cross-linking [19-21].

Early in the wound healing process, there is an inflammatory phase [19-21] which results in an invasion of the wound site(s) by large amounts of polymorphonuclear leukocytes and macrophages which result in the production, and activation, of reactive oxygen species (ROS), a major defense mechanism [19-21]. However, excess amounts of ROS can inhibit cell migration and proliferation, and is known often to promote severe tissue damage, preventing wound healing. But, our present studies and those published by our group previously, demonstrate that HDFx attenuates, greatly, the production of lipid

peroxidation as seen from the reduced levels of MDA. Moreover, our findings of HDFx-reductions in the production of SOD woud suggest that our novel biologic protein lowers oxidative stress and is probably a major factor in lowering the inflammatory response, thus insuring better and faster healing of the wounds.

The fact that HDFx improves microcirculatory blood flow after tissue trauma [2] probably also plays an important role in the wound healing process. Improvement of local blood flow to injured areas is known to promote wound healing [19-21]. HDFx is unique in that it improves vasomotion and vascular reactivity of the microcirculatory blood vessels after trauma and tissue injury [2].

HDFx, from the results, presented herein, appears to possess a number of regenerative qualities. For example, it clearly results in renewal of damaged tissue more rapidly than the host's own powers; and it renews the skin identically to the original of the host. Whether HDFx promotes regeneration of the damaged skin via stimulation of precursor cells or stem cells or both will have to await further studies.

At the present time, and into the future, the U.S. and NATO armed forces, as well as the civilian populations world-wide, will be at considerable risk from vesicant agents, blast injuries, and diverse microbes which often result in difficult-to-treat chronic infections at great financial costs. These often, untreatable wounds-infections require multiple, continuous surgeries which result in major lifelong impairments in arms, fingers, legs and other body parts. The implementation and use of HDFx could attenuate these chronic wounds and infections , thus preventing loss of use of these body parts and ,at the same time, save billions of dollars in health care costs.

Conclusion

In conclusion, our present report on accelerated and improved wound healing presents provocative evidence, when viewed in light of our previous recent findings on the beneficial effects of HDFx on infectious microorganisms, diverse types of circulatory shock, and trauma, to strongly suggest that HDFx should be considered as a serious contender for the treatment of wounds and the sequelae of events (e.g., infections, shock, gangrene, amputations) which often occur in battlefield casualties, disaster victims, and prolonged surgical procedures, particularly in aged and debilitated victims. As HDFx is a conserved protein molecule (35-40 KDa in size) found in a variety of animals so far, e.g., rats, mice, guinea pigs, and rabbits [1], and synthesized in macrophages and natural killer cells [1], it more than likely exists in human subjects.

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