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HYPERBARIC OXYGEN EFFECTS ON BROWN RECLUSE SPIDER (*LOXOSCELES RECLUSA*) ENVENOMATION IN RABBITS

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G. M. STRAIN, T. G. SNIDER, B. L. TEDFORD and G. H. COHN. Hyperbaric oxygen effects on brown recluse spider (*Loxosceles reclusa*) envenomation in rabbits. *Toxicon* **29**, 989–996, 1991.—Human loxoscelism was modeled in albino rabbits by injection of brown recluse spider (*Loxosceles reclusa*) venom, and the effects of daily or twice-daily hyperbaric oxygen treatment on wound healing were investigated. Lesions similar to those seen in humans were produced in rabbits by intradermal injection of 200 μl of a venom extract (0.21 μg protein per μl), including edema and erythema, ischemia and cyanosis in the first 12 hr, extensive purpura by 24 hr, and crateriform ulcer formation by day four, with induration and eschar formation. Hyperbaric oxygen treatments, consisting of two atmospheres absolute (2 ATA) for 60 min, were applied daily ($n = 8$) or twice daily ($n = 8$), while control animals ($n = 8$) received no treatment. Treatments were initiated 72 hr after venom injection (day 3) to duplicate typical clinical treatment delays, and were administered for seven consecutive days. No significant effects of hyperbaric oxygen treatment on lesion healing were seen as measured by lesion area. However, histologic evaluation of wound tissue collected at euthanasia on day 24 showed clear differences between rabbits receiving twice-daily treatments and those receiving daily or no treatment. The former showed complete re-epithelization or slight ulceration, while the latter usually had necrotic cavities extending into the dermis, with myonecrosis and inflammatory cell accumulation. Thus, no superficial differences were seen between groups, but twice-daily treatments resulted in enhanced recovery at the histologic level.

INTRODUCTION

BITES of humans by the brown recluse spider (*Loxosceles reclusa*) result in development of necrotic ulcers in 10% of the victims that may take 4–12 weeks to heal. Ulcer formation follows a predictable, although relatively painless, course, consisting of erythema, wheal, blister, local edema, purpura, cyanosis and ischemia by 24 hr and necrosis and crateriform ulcer in 3–4 days (MORGAN, 1984). Treatment efforts are mostly palliative, including ice application, antibiotics, aspirin and dapsone (KING and REES, 1986); steroids, local

incision and heat are ineffective or contraindicated. Because initial local reactions are unremarkable and frequently resolve without serious sequelae, subjects with developing necrotic ulcers are generally not seen for treatment until 72 hr or more after the bite.

Experimental studies with rabbits have suggested that the necrotic lesion of loxoscelism results from intravascular coagulation, secondary to polymorphonuclear leukocyte adhesion to capillary walls (BERGER *et al.*, 1973; GEREN *et al.*, 1985; C. R. GEREN, personal communication 1989). Venom has been shown to exhibit primary sphingomyelinase activity, with lesser protease, lipase, nonspecific hydrolase and direct hemolytic activity (REKOW *et al.*, 1983). Based on the findings of intravascular coagulation and on empirical bases, hyperbaric oxygen therapy has been suggested in such cases (MORGAN, 1984; MYERS, 1986; SVENDSEN, 1986). A recent publication (SVENDSEN, 1986) reported an uncontrolled study of hyperbaric oxygen treatment of six clinically-diagnosed brown recluse bites in humans first seen 48 hr to 7 days after envenomation. Treatments of 60 or 90 min at 2 atmospheres absolute (ATA), equivalent to a pressure of 15 psi, were given twice-daily, with subjects receiving 1–6 total treatments; uneventful prompt healing without skin sloughing or significant scarring was reported for every case.

In the present study, the effect of hyperbaric oxygen treatment was objectively evaluated in a rabbit model of brown recluse spider envenomation, comparing daily and twice-daily hyperbaric oxygen treatments of 60 min at 2 ATA. Rabbits were chosen due to the high resistance of mice and rats to the development of necrotic skin lesions and the high incidence of lethality in guinea pigs at doses producing lesions (MORGAN, 1969; BABCOCK *et al.*, 1981a).

MATERIALS AND METHODS

Subjects

Adult male New Zealand albino rabbits (Hazelton Research Laboratories, Denver, PA, U.S.A.), ($n = 24$) were caged individually in an environmentally controlled room under a 12:12 lighting schedule, with rabbit chow and water available *ad libitum*. The rabbits were anesthetized (ketamine, 50 mg/kg, and xylazine, 10 mg/kg, i.m.) one day prior to venom injection for shaving the injection site to minimize the presence of skin abrasions at injection time. All appropriate animal care and use guidelines were observed in the study.

Venom isolation and injection

The venom extract was a kind gift from Dr Collis R. Geren, University of Arkansas, Fayetteville, U.S.A. Venom preparation was performed as previously described (BABCOCK *et al.*, 1981*b*). Wild-captured spiders were frozen, and the entire venom apparatus was dissected and homogenized in 20 mM Tris-buffered saline (pH 7.4). Insoluble material was removed by centrifugation, resulting in a final venom protein concentration of 0.21 $\mu g/\mu l$ with an approximate i.p. LD_{50} of 25μg. Venom was prepared for injections in three separate batches. A dose of 200 μl of venom extract was injected intradermally in anesthetized rabbits in a shaved area of the right flank, 2–4 cm off midline, taking care that no venom leaked from the injection site.

Hyperbaric oxygen treatment

The injected rabbits were separated into three groups: contols ($n = 8$), treatment group I ($n = 8$) which received daily hyperbaric oxygen, and treatment group II ($n = 8$) which received twice daily hyperbaric oxygen. Rabbits were held in separate plastic cages for treatment in an Air Force Type II animal transfer hyperbaric chamber (0.5 m diameter, 1.3 m length). An initial 5 min flush of pure oxygen at 100 liters/min, 1 ATA, to purge other gases from the chamber was followed by pressurization to 2 ATA. During the 60 min treatment period a continuous O_2 flow of 5 liters/min was maintained to eliminate CO_2 buildup. Treatment was initiated on day 3 (injection day = day 0), 72 hr after injection, to duplicate typical clinical presentation delays. Daily (group I) or twice-daily (group II) treatments were given for seven consecutive days (days 3 through 9).

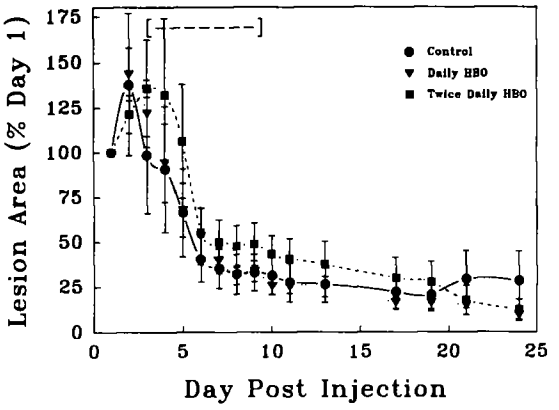

FIG. 1. BROWN RECLUSE SPIDER VENOM LESION SIZE AS A FUNCTION OF TIME AFTER INJECTION. Measured lesion areas of rabbits ($n = 24$) injected intradermally with brown recluse spider venom as a function of time after injection (day 0), expressed as a percentage of day 1 lesion area (mean ± SEM). Treatments consisted of hyperbaric oxygen at 2 atmospheres absolute for 60 min once daily (triangles) or twice daily (squares); control animals (circles) received no treatment. No significant effect of hyperbaric oxygen treatment was seen on lesion size.

Analysis of lesions

Lesions were photographed on days 1–11, 13, 17, 19, 21 and 24. Lesion area in cm^2 was quantitated from photographs using a digitizing tablet system (Sigmascan, Jandel Scientific Corp.). Statistical differences between control and treatment groups with time were determined by repeated measures analysis of variance, with a $P = 0.05$ level of significance. Rabbits were killed with CO_2 on day 24, after which the complete lesion and a comparable sample of normal skin from the contralateral flank were excised and immediately placed in neutral buffered formalin. Tissue sections were processed for paraffin embedding and 4–6 μm sections were stained with hematoxylin and eosin or with a modified trichrome stain for connective tissue (CROWDER, 1983).

RESULTS

Lesion development

Within the first 12 hr the injection sites followed a pattern of initial edema and erythema, ischemia, cyanosis and initial tissue destruction. Extensive purpura was present by 24 hr, in a pattern indicating considerable gravity flow of venom which in some rabbits extended to the ventral midline. The purpura and erythema increased through 48 hr. By day 4 crateriform ulcer formation had begun, with induration and eschar formation. Eschar formation was completed by days 5–6; partial sloughing began during the next few days and continued over weeks 2–4, depending on the initial ulcer size and the extent of scar formation. Lesion area ranges (cm^2) on days 1 through 3 for all 24 rabbits were 1.35–18.41 (7.92 ± 1.13, mean ± SEM), 0.23–31.03 (10.18 ± 1.85), and 0.18–30.49 (9.12 ± 1.77), respectively, decreasing in size thereafter. Large animal-to-animal variability in lesion size was seen both within and between venom batches.

Hyperbaric oxygen effects

Animal skin demonstrated an increased pink color immediately after hyperbaric oxygen treatment. No deleterious effects of hyperbaric oxygen were observed, including no distress during or after hyperbaric treatments. Lesion areas increased in size through day 2, after which time they began to decrease. No difference in absolute lesion size between groups was seen by day 8. However, considerable differences between groups in lesion

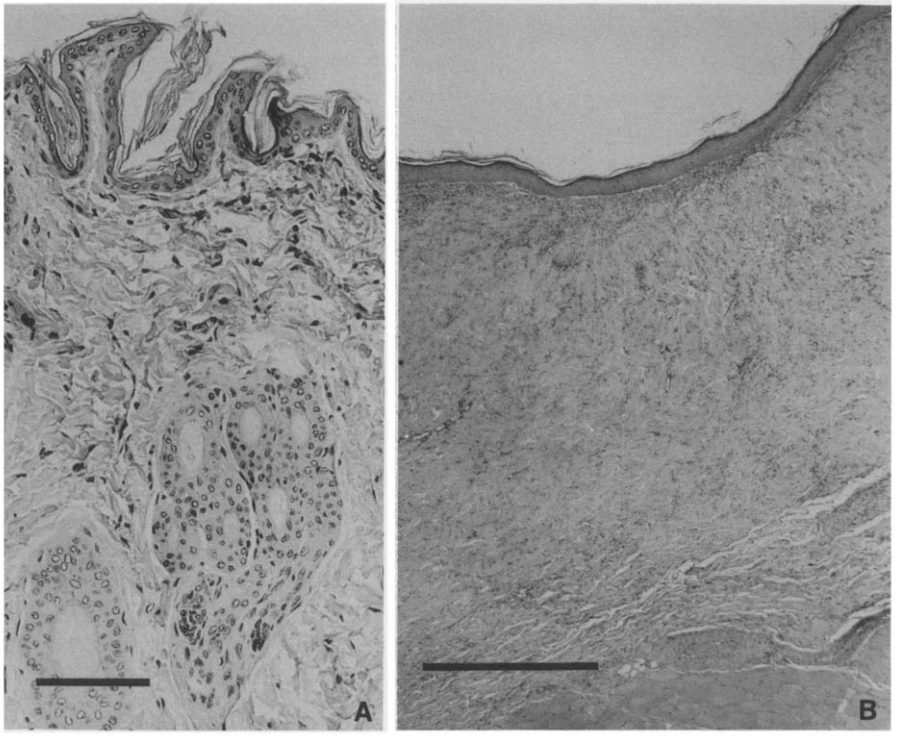

FIG. 2. COMPARISON OF NORMAL UNINJECTED RABBIT SKIN WITH VENOM-INJECTED SKIN AFTER TWICE DAILY HYPERBARIC TREATMENT.

A. Normal rabbit skin from the non-injected left flank demonstrates thin layers of keratin, thin rugose epidermis, loose dermal connective tissue, and adnexal structures. Haematoxylin and eosin staining. Calibration: 100 μm. B. Venom extract (200 μl) was injected intradermally in anesthetized rabbits in a shaved area of the right flank, 2–4 cm off midline. The injection site from a rabbit that received twice daily hyperbaric treatment shows re-epithelization, absence of adnexal structures with proliferation of fibroblasts and fibrocytes, and minimal cellular inflammatory response, with normal subcuticular muscle. Haematoxylin and eosin staining. Calibration: 1 mm.

size were apparent before the initiation of hyperbaric oxygen, so lesion sizes were expressed as a percentage of the size on day 1 to facilitate comparisons (Fig. 1). Statistical analysis of the normalized data by multivariate repeated measures analysis of variance demonstrated a significant effect of time ($P < 0.0001$) for each group, but no significant effect of treatment ($P = 0.292$).

Tissue evaluation

Histological evaluations were performed on tissues collected 24 days after initial injection with the reader blind to the treatment status. On this basis, seven of the eight rabbits in treatment group II were correctly identified as having hyperbaric treatment, whereas only four of the sixteen rabbits receiving either no treatment or daily hyperbaric treatment (treatment group I) were correctly sorted on the basis of histologic changes.

The skin lesions of the rabbits in treatment group II were characterized by complete re-epithelization or slight centrally located ulceration with no or lymphocyte or plasma cell

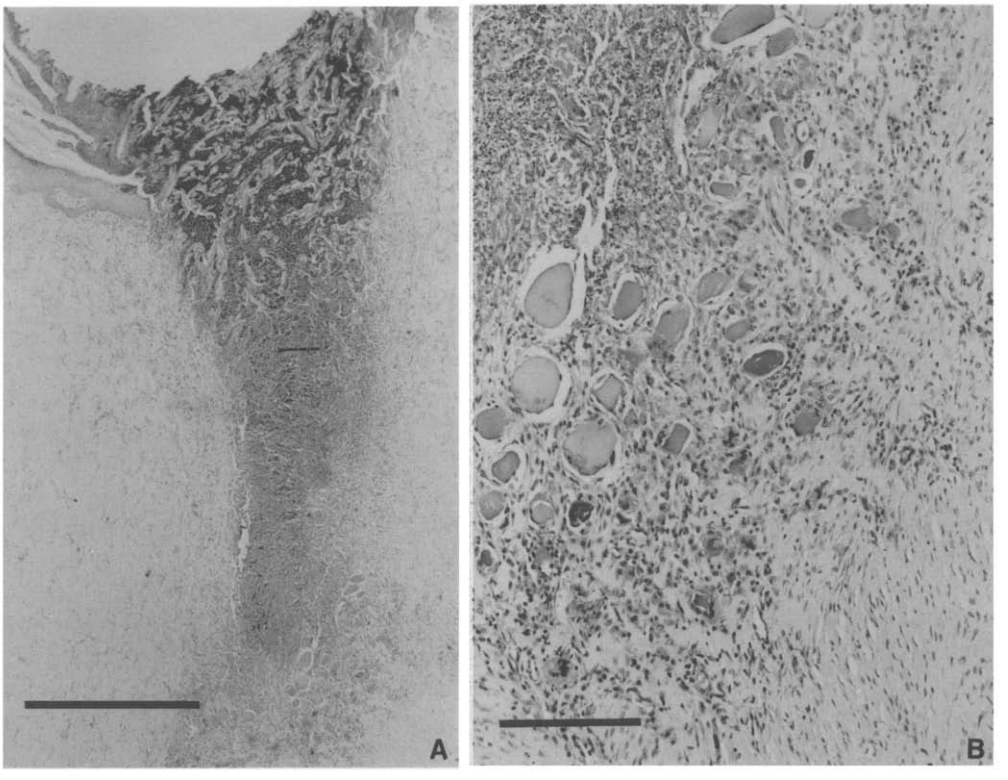

FIG. 3. MICROSCOPIC APPEARANCE OF LESIONS AT BROWN RECLUSE VENOM INJECTION SITES. A. Injection site (right flank, 2–4 cm off midline) from a rabbit receiving no hyperbaric treatment, showing a necrotic zone filled with heterophils and cellular debris extending through the dermis and covered with a thick fibrinonecrotic crust. Haematoxylin and eosin staining. Calibration: 1 mm. B. Magnification of lower right corner of A, showing extension of the inflammatory process from the dermis into the subcuticular muscle, with myonecrosis and inflammatory cell accumulation. Haematoxylin and eosin staining. Calibration: 200 μm.

infiltration. Crusts were commonly present overlying a thin layer of epithelium. Adnexal structures and normal dermal connective tissues were replaced by irregular bundles of fibroblasts and fibrocytes (Fig. 2B). Dense irregular collagen bundles were demonstrated with the trichrome stain. Extension of the tissue reaction to the subcuticular muscle was rarely present. By contrast, normal skin displayed thin layers of keratin, a thin, slightly rugose epidermis, loose dermal connective tissue, and adnexal structures (Fig. 2A).

Seven of the eight treatment group I rabbits which received daily hyperbaric treatments had severe ulceration and necrosis extending into the dermis. Degeneration of collagen, and focal and diffuse infiltrations of heterophils and macrophages were common lesion components. Re-epithelization with prominent hyperplasia and acanthosis was generally limited to a narrow zone bordering the ulcer.

Two of the three control rabbits receiving the first batch of venom and three of the five receiving the second batch of venom had partial lesion resolution; the remaining three control rabbits showed extensive lesion development. There was a thick fibrinonecrotic crust overlying a necrotic zone filled with heterophils and cellular debris extending

through the dermis (Fig. 3A). The inflammatory process involved the subcuticular muscles with myonecrosis and inflammatory cell accumulation, including macrophages, lymphocytes and plasma cells (Fig. 3B).

DISCUSSION

The pattern of necrotic ulcer formation in humans has been reported to consist of erythema, wheal, blister, local edema, purpura, cyanosis and ischemia by 24 hr, and necrosis and crateriform ulcer in 3–4 days (MORGAN, 1984). A similar pattern was observed in the rabbit. A high variability in lesion size was observed in the rabbits after uniform venom doses. This was most likely a reflection of the extent of intradermal and subcutaneous venom dispersion, since lesions were largest in animals where a clear gravity-induced spread of the venom occurred. Although anecdotal reports have described remarkable recoveries in human bites (SVENDSEN, 1986), there was no beneficial effect of daily or twice-daily hyperbaric treatments on rabbit experimental envenomation when assessed on the basis of lesion surface area. However, a clear treatment effect after twice-daily hyperbaric oxygen was seen at the histological level.

The mechanism of ulcer formation by brown recluse venom toxin is not currently understood. Two theories are espoused, one suggesting initial capillary thrombosis followed by a polymorphonuclear leukocyte-mediated inflammatory response (BUTZ *et al.*, 1971; BERGER *et al.*, 1973), while the other suggests an initial polymorphonuclear leukocyte adhesion to capillary walls followed by thrombi formation (SMITH and MICKS, 1968; GEREN *et al.*, 1985). Studies with isolated venom components have shown a Ca^{2+}-dependent platelet aggregation *in vitro*, with an unknown plasma component being necessary for the effect (REES *et al.*, 1988). However, *in vivo* experiments have shown polymorphonuclear leukocyte adherence to capillary walls as early as 1 hr after venom injection, while hemorrhaging was not present until 4–8 hr, when capillary walls had broken down and leukocytes had diapedesed (GEREN *et al.*, 1985; C. GEREN, personal communication 1989). Depletion of polymorphonuclear leukocytes with nitrogen mustard blocks reaction to the venom (SMITH and MICKS, 1970), and the anti-leprosy medication dapsone, which is used in the treatment of bites, blocks polymorphonuclear leukocytes. Dapsone use many hours after the spider bite must have limited value, however, since polymorphonuclear leukocyte-induced capillary damage is well-advanced at that time. The mechanism whereby polymorphonuclear leukocytes are activated is not known. Brown recluse venom activates complement in plasma (KURPIEWSKI *et al.*, 1981), but polymorphonuclear leukocyte accumulation is complement-independent, possibly resulting instead from tissue macrophage factors (ISSEKUTZ *et al.*, 1987).

Hyperbaric oxygen effects on wound healing after initial tissue damage may be mediated by two mechanisms. The first is through its role in enhancing angiogenesis. Tissue regeneration requires sufficient capillary ingrowth, which in turn relies on collagenous support. Wound centers have very low oxygen tensions (REMENSNYDER and MAJNO, 1968), which provides the 40 mm Hg or greater oxygen gradient necessary for wound healing angiogenesis (KNIGHTON *et al.*, 1981). Macrophages in the wound dead space produce factors which attract fibroblasts. However, fibroblasts at the wound margin require a tissue PO_2 of 40–55 mm Hg for replication and production of the collagen matrix necessary for capillary endothelial cell growth and extension into the wound. Periodic hyperbaric oxygen produces the elevated oxygen tension necessary for fibroblast activa-

tion by increasing the distance oxygen diffuses from existing capillaries, while normobaric periods continue the stimulatory oxygen gradient necessary for continuation of healing (KNIGHTON *et al.*, 1981). The second mechanism by which hyperbaric oxygen may influence the healing of brown recluse venom lesions is through a direct inactivating effect on the venom or one of its components. It has been shown that hyperbaric oxygen inactivates enzymes containing sulfhydryl groups (HAUGAARD, 1971), and it has been suggested (SVENDSEN, 1986) that enzyme inactivation has occurred when clinical subjects obtain beneficial effects from hyperbaric oxygen treatments. However, further study will be required to confirm this suggestion.

Based on lesion appearance and the time course of recovery, the present rabbit model of brown recluse envenomation appears to provide an excellent tool for investigations of lesion pathogenesis as well as therapeutic interventions, despite a high variability in the size of lesion produced. If this lesion is a valid model for human loxoscelism, the results of this study suggest that any benefit to be derived from hyperbaric therapy requires twice-daily treatments. Intervention at the earliest possible opportunity is also mandated to minimize tissue damage and institute angiogenesis into the lesion for its resolution.

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