Healing action of nerve growth factor on lameness in adult goats

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Summary. - We investigated the effect of nerve growth factor in goats affected by lameness, a condition that is associated with cutaneous ulcers, abscesses and granulomas. Lesions were treated daily with 1 µg of nerve growth factor (NGF), dissolved in 100 µl of sesame oil or with sesame oil only. Healthy controls were treated with sesame oil only for periods ranging from two to four weeks and behavioral, biochemical and histopathological conditions were evaluated. Topical application of NGF, but not sesame oil, promoted ulcer healing in all goats within two-three weeks of treatment. The effect of NGF was to re-establish in about 60 days normal locomotion and indirectly to improve feeding behavior and milk production. These findings suggest that NGF might be clinically useful for healing damaged skin in goats and confirm previous studies on human corneal ulcers.

Key words: ulcer, NGF, lameness, skin, behavior, goat.

Introduction

In numerous farm animals and particularly in goats and sheep, lameness is associated with the presence of cutaneous ulcers, abscesses, and granulomas in the interdigital and sole area of the anterior and posterior feet [1-4]. Footpain caused by lameness impairs locomotor activity, alters feeding behavior and decreases body weight [1, 5]. Several factors, including housing and flooring, nutritional, environmental, genetic and contagious factors are believed to be involved in the pathogenesis of lameness [3]. Indeed, lameness in goats may be induced by anaerobic gram-negative bacteria such as Dichelobacter nodosus. At present, the mechanism(s) implicated in this condition remain largely unknown, and the available therapies do not promote complete healing of lesions. In adult female goat lameness can cause decreased milk production, poor fertility, and affect the care of offspring. Therefore, the economic costs are very high and there is a pressing need to understand the mechanism(s) involved in ulcer formation and to identify pharmacological compounds that can be used to promote healing. Thus, the identification of any molecule that is able to block the lesions and/or to promote healing would be of therapeutic interest. It has been reported that topical application of nerve growth factor (NGF), a polypeptide exerting a variety of biological activities on neuronal and non-neuronal cells [6-8] is able to stimulate healing in human corneal and pressure ulcers [9, 10]. Other studies carried out in animal models have also shown that NGF accelerates the rate of wound healing, exerts therapeutic action on damaged peripheral nerve and promotes tissue repair in ocular inflammation [11-14]. These and other studies indicate that the effects of NGF are mediated by NGF receptors present on cutaneous cells, such as fibroblasts, keratinocytes, mast cells and immune-competent cells [12-16]. Taken together, these observations raised the question whether NGF can exert a similar reparative action on cutaneous lesions of domestic and/or farm animals. To test the validity of this hypothesis, we investigated the effect of topical

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Riassunto (La molecola NGF promuove la guarigione della pododermatite nella capra adulta). - La pododermatite (PDD) è una malattia molto diffusa tra caprini, ovini e bovini, caratterizzata da una progressiva distruzione necrotica del derma mentre il nerve growth factor (NGF) promuove la guarigione di ulcere oculare e cutanee nell’uomo. Partendo da questi dati sperimentali sedici capre adulte affette da PDD sono state tratte con 1µg al giorno di NGF per tre settimane. Otto capre affette da PDD sono state state trattate con il solvente e utilizzate come controllo. Otto capre sane sono state trattate con il solvente come ulteriore gruppo di controllo. I risultati dimostrano che l’NGF induce la guarigione delle ulcere podali mentre tale effetto non si nota nei controlli. I nostri studi suggeriscono un potenziale ruolo terapeutico dell’NGF nella PDD dei caprini.

Parole chiave: ulcera, NGF, pododermatite, cute, comportamento, capra.
application of NGF on foot lesions occurring in lameness goats. We report here that purified NGF is a marked promoter of peripheral wound healing in goats suggesting that this molecule may have therapeutic potential to heal foot lesions of farm animals.

**Materials and methods**

**Animals**

Our present studies are based on 24 adult female goats (*Capra hircus ionica*) affected by lameness in the anterior or posterior feet unresponsive to the available therapy. Sixteen goats were treated with NGF dissolved in sesame oil and 8 goats with sesame oil only. Eight healthy goats were used as further controls and treated with sesame oil only. Dead tissue was trimmed with the goat standing and restrained by a collar tied to the pen [17] after mild local carbocaine anesthesia to reduce animal pain and suffering. Sole trimming removed the thick stratum of cornified cells in order to expose the dermal and the granular layers and allow the lesions to be classified and treated. For the classification of lesions we used previously described general criteria [5, 18]. All adult goats were provided by a farm located in South Italy (Azienda Agricola Cuccumella, Cardinale, Reggio Calabria, Italy, written informed consent was obtained from the farmer). Their mean age was 18 months (ranging from 17 to 19 months) and they were housed during our experiments in straw yards in a group of about 24 animals. Goats were fed with grass silage and maize ad libitum. The criteria for selecting the goats were: a) walking impairments; b) presence of skin or sole ulcers for at least 2 months (Table 1); c) and no spontaneous healing during this period. All lameness goats had reduced appetites. Lesions were treated topically with NGF. Clinical study protocol and procedures were conducted in conformity with the Intramural Committee and Institutional Guidelines in accordance with National and International laws (EEC Council Directive 86/609, Official Journal of the European Communities L 358, 1, December 12, 1987).

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<td>Ulcerative lesion</td>
</tr>
<tr>
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<td>Granulomatous lesion</td>
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</table>

**Behavioral scoring of lameness goats**

To evaluate the effect of topical application of NGF, goats were observed between 7:00 am and 9:00 am, both before and after three weeks from the NGF treatment for their standing position, walking behavior, as well as body weight and milk production. Standing behavior was monitored using a 2 min test to measure the time spent with 4 feet on the ground. Walking was measured as the time necessary to cover a distance of 30 meters along an enclosed path to reach the nearest available food source in the pen for a 12 h food deprived animals. Since lameness is known to affect feeding behavior, body weights and milk production were measured in healthy goats treated with sesame oil and NGF-treated goats at the beginning and at the end of NGF treatment. All behavioral measurements were scored by two observers unaware of the group assignment of the animals.

**NGF purification and treatment**

Highly purified NGF was prepared in our laboratory from adult male mouse submaxillary glands following the method described by Bocchini and Angeletti [19], and the biological activity of each preparation was tested with an *in vitro* bioassay [20] to ensure equal activity. Purified NGF was dissolved in sesame oil (Fisher Chemical Co, USA) and applied topically on a dressing for 2 h. Ulcers were treated after foot trimming to remove scar and non-vascular sole tissues with 100 µl of solution containing 1 µg of NGF. NGF was applied on the foot lesioned area once a day at about 10 am, controls were treated topically with 100 µl of sesame oil only. Animals were treated daily for 3 weeks.

**Morphological evaluation**

Skin lesions were observed every day, but morphological analysis was carried out only at the beginning and at the end of the treatment to avoid unnecessary animal suffering. Small pieces of tissues were cut from the interdigital and sole regions of lameness goats after a mild carbocaine anesthesia. Lesioned tissues were removed and processed separately for histological and immunohistochemical analysis. They were then fixed immediately in 2% paraformaldehyde in 0.1M phosphate buffer containing 0.5% parabenzoquinone (Sigma-Aldrich, St. Louis, USA) and stained with hematoxylin-eosin or toluidine blue for routine histological examination. Pieces of tissues taken after a mild carbocaine anesthesia from the same region of healthy goats were similarly processed and used as controls. Parallel sections were immunostained for the localization of NGF and NGF-receptors.
NGF determination

The levels of endogenous NGF were measured in the interdigital skin tissues and in the sole of healthy controls and in lameness goats before the NGF treatment by a highly sensitive two-site immunoenzymatic assay which recognizes human and murine NGF following methods previously described with minor modifications [20-22]. Control fresh tissues (the same healthy animals of the behavioral tests) were obtained from slaughter-house immediately after the sacrifice. The optical density was measured at 575 nm using an ELISA reader (Dynatech, MR 5000, PBI International, Germany), and the values of standards and samples were corrected by taking into consideration the non-specific binding. The recovery of NGF during the assay procedure [21] was estimated by adding a known amount of highly purified NGF (NGF 2.5S in extraction buffer, see above, used as standard in a range of 0.015 pg/ml-1ng/ml) to the samples or to the extraction buffer, as internal control. The yield of the exogenous NGF was calculated by subtracting the amount of endogenous NGF from the value of endogenous plus exogenous values. Under these conditions, the NGF recovery was over 90%. NGF concentration is expressed as pg of NGF/mg of proteins and all assays were performed in triplicate.

Immunohistochemistry

For histological, immunohistochemical and immunoenzymatic analysis we used tissues with ulcers with or without granulomas. After fixation in paraformaldehyde, tissues were cryoprotected in PBS containing 30% sucrose for 3 days, then mounted on a freezing microtome and 30 µm transverse sections were cut and put on gelatin coated slides. Sections were preincubated with 2% BSA and 10% normal preimmune serum in PBS solution containing 0.5% Triton X-100 for 1 h and then incubated overnight at 4 °C with polyclonal antibody anti-NGF (1/1000) or anti-high affinity NGF receptor TrkA from Santa Cruz, USA (diluted 1/100). After a brief rinse in PBS, sections were incubated for 1 h at room temperature with biotinylated secondary IgG antibody (1:300) and then the avidin biotin complex kit conjugated to horseradish peroxidase following the description of the manufacturer (Vector Laboratories, Burlingame, CA, USA). The staining specificity was assessed by omitting the specific antibody. Sections were then rinsed in PBS, mounted in glycerol-PBS solution, and examined with a Zeiss Axiophot microscope for a qualitative evaluation.

Statistical analysis

Biochemical and behavioral data were analyzed by analysis of variance (ANOVA) considering the NGF treatment as between-subject factor by using the StatView 5.0 package for the Macintosh. When statistical differences were obtained (p < 0.05), post hoc comparisons within logical sets of means were performed using the Tukey’s test.

Results

Macroscopic observations

Lameness goats used in our studies were unable to walk properly, displayed severe impairment of locomotor activity, and were characterized by the presence of ulcers with or without granulomatous tissues in the interdigital skin and sole regions. Granulomas appeared as vascular nodules within or protruding from the sole. Nodules were haemorrhagic and up to 1 cm in diameter. Lesions were also associated with necrotic soles and with a distinct and characteristic odor.

Skin lesions and NGF distribution

As shown in Fig. 1 the concentration of NGF in the interdigital cutaneous tissues was lower in lameness goats than healthy controls (p < 0.05). To assess whether NGF is locally produced and whether resident cells are receptive to the action of NGF, interdigital and sole tissues of healthy controls and lameness goats before the NGF treatment were immunostained with anti-NGF or anti-TrkA. The result showed marked NGF immunoreactivity, mainly localized around the granulomas, while TrkA-immunoreactivity was found in the cutaneous tissue. The interdigital skin of lameness goats before the topical NGF treatment expressed high affinity NGF-receptors (Fig. 2 A) suggesting that dermal

Fig. 1. - Amount of NGF in the interdigital skin tissues of lameness goat before NGF treatment and healthy controls. Note that the level of NGF in lameness goats is statistically lower as compared to healthy goats (p < 0.05 in the ANOVA). Data represent mean levels ± SEM. Asterisks indicate significant between-group differences (**p < 0.01).
cells of goats respond to the action of NGF. Likewise, dermal cells of the sole of both healthy control and lameness goats before NGF treatment also express TrkA (data not shown). The NGF-receptor immunopositivity is specific, since adjacent sections processed by omitting the primary antibody stained negatively (Fig. 2 B).

Effect of topical application of NGF

To investigate whether altered amounts of NGF are implicated in lameness associated wound lesions, ulcers and granulomas were treated with purified NGF. The results demonstrated that topical application of NGF on interdigital or sole lesions induced tissue healing. On the contrary, lameness goat treated with sesame oil only did not show tissue healing and any behavioral recovery. Fig. 3 shows the sole of a goat affected by lameness before (A) and after (B) topical treatment of NGF for three consecutive weeks. Daily observation indicated that the action of NGF was evident 3-4 days after NGF administration, while complete healing occurred after three weeks from the NGF treatment. Three weeks after NGF treatment sole lesions were no longer present and the morphological structure of the damaged tissue was comparable to that of control tissues in 11 treated goats whereas 5 animals still presented small scars (Table 2). The healing effect is due to NGF, since NGF pretreated with NGF-antibodies to inhibit its biological activity, was unable to promote ulcer healing (data not shown).

Healing action of NGF on locomotor activity

To evaluate the locomotion of healthy controls and lameness goats, we tested the walking ability of these animals before and after topical administration of NGF (Fig. 4). The goats affected by lameness took significantly more time to cover 30 meters when compared to healthy animals or lameness goats treated with NGF (p < 0.05). Specifically, three weeks after topical NGF application, lameness goats had recovered nearly 60% of their walking ability. The effect of NGF on locomotion was also confirmed by the standing behavior of the lameness goats treated with NGF for three weeks. The goats displayed a normal standing posture (p < 0.05). Moreover, the healing action of NGF on foot ulcer and locomotor activity was associated with an improved feeding behavior and milk production (p < 0.05). Complete recovery in both body weight and behavior in lameness goats treated with NGF was observed 60 days after the beginning of the NGF treatment.

Fig. 2 - Pictures showing the skin of the interdigital area of lameness goats before NGF treatment expressing positivity for the high-affinity NGF-receptor (TrkA) with (A) or without (B) the TrkA antibody. The presence of NGF-receptor suggests that the skin of the interdigital area has the ability to respond to the action of NGF. Scale bar = 75 µm.
NGF IN LAMENESS

Discussion

The results of the present study show that goats affected by lameness completely recovered in about 60 days reestablishing feeding, milk production and behavior. NGF is the first and the best characterized member of a family of neurotrophic factors exerting a crucial role on peripheral and brain neuropathies in laboratory animals and in humans [7]. The present study demonstrates that NGF is also found in goat foot tissue and that the interdigital skin and the dermal sole tissues express NGF-receptors. Our findings also show a decrease in NGF in the ulcers and an increase in the granulomas when compared to their respective controls. The mechanisms leading to wound healing are complicated and involve the participation of a variety of different cell types, such as fibroblasts, keratinocytes, eosinophils, neutrophils, macrophages and lymphocytes. Because these cells are not only able to produce NGF, but are also receptive to the action of NGF [14], it is highly likely that the reparative action depends on the amount of NGF present around the damaged tissues. Thus, the endogenous concentration of NGF may act as an early attempt to promote the recovery of injured skin. Indeed, available evidence indicates that the up-regulation of NGF release immediately after injury is not sufficient to block cell damage and/or stimulate tissue repair but requires exogenous administration of NGF [8, 10, 23]. The effect of NGF on ulcer healing is consistent with our previous findings in humans showing that topical application of NGF induces healing in corneal and pressure ulcers [9, 23].

The NGF effect on goat ulcers is specific since cutaneous cells express NGF receptors and, NGF preabsorbed with anti-NGF antibody, which blocks NGF biological activity, is unable to promote healing. Indeed, TrkA expression in the skin of the interdigital area of lameness goats is present only in the surface at the level of keratinocyte action suggesting that NGF is essential for cutaneous wound healing [9, 14]. High levels of circulating anti-NGF antibody in adult goats cause small skin ulcers localized in particular in the neck and at the back of the ears (Costa et al., in preparation) as previously shown in rodents [24].

Lameness is a condition affecting farm animals, that is of great clinical importance in veterinary medicine not only for the health and welfare of animals, but also it results in loss of revenue for farmers. Despite the limited number of subjects used, one important implication of our results is that NGF may have therapeutic potential for foot lesions in goats affected by lameness. Indeed, on the basis of these and of our previous observations in human ulcers it is reasonable to hypothesize that topical application of NGF may represent a useful pharmacological approach to reduce and/or prevent the development of peripheral ulcers not only in lameness goats, but also in other farm animals [25-28].
Table 2. - Chronological sequence of the NGF administration and outcome of each recorder case. Animals 1-16 were treated with NGF dissolved in sesame oil. Animals 17-24 were treated with sesame oil only.

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NGF: nerve growth factor.
Acknowledgements

This study was in part supported by Fondazione CARISBO, Bologna, Italy to Luigi Aloe and by Plurifondo CNR, Biotech, Subproj 5.

Received on 26 November 2001.
Accepted on 2 May 2002.

REFERENCES


Fig. 4. - Standing ability, time to cover 30 meters, body weight and milk production in healthy and lameness goats before and after NGF treatment. This result suggests that the effect of NGF on the recovery of the lesioned foot is that lameness animals spend more time in eating producing also more milk (p < 0.05). Data represent mean levels ± SEM. Asterisks indicate significant between-group differences (*p < 0.05, lameness goats vs healthy goats).


