Defocused CO$_2$ Laser Irradiation in the Rehabilitation of Horses

An Experimental and Clinical Study

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Abstract


There is an increasing interest in the use of physical medicine in rehabilitation of animals. The main goal in rehabilitation is to regain best possible physical function after illness or injury, by use of different physical modalities. The aim of the present investigation was to study the photothermal effects of defocused CO₂ laser irradiation on equine tissue, one modality used in the rehabilitation of horses. The thesis comprises studies on the effect of irradiation on temperature, blood flow, morphology, concentration of anti-inflammatory and pain modulating mediators, and finally, lameness due to traumatic arthritis of the fetlock joint.

Three experimental studies revealed that defocused CO₂ irradiation (91 J/cm²) causes a moderate to vigorous heating effect (3-6 °C) in superficial tissues, with a concomitant increase in blood flow, detected by Laser Doppler Flowmetry. Mild to severe dose-dependent morphological changes were detected in the skin after irradiation with doses ranging from therapeutic to near-surgical (91-450 J/cm²). A clinical study demonstrated a decrease in the degree of lameness in both groups of lame horses after irradiation. No statistical difference was detected between lame horses treated with laser or placebo, evaluated by conventional lameness examination and accelerometer technique. Nor was there a difference in the concentration of inflammatory mediators such as substance P and PGE₂, or the opioid Met-enkephalin-Arg-Phe in synovia. A higher concentration of Met-enkephalin-Arg-Phe was measured in sound horses compared to horses with traumatic arthritis.

In conclusion, the present thesis reveals that irradiation with defocused CO₂ laser causes a moderate to vigorous heating effect in superficial tissue, and a marked increase in blood flow. The increase in temperature was of such intensity that there is a potential risk of thermal injuries to the skin. The results also suggest that treatment with defocused CO₂ laser is not statistically better than placebo at reducing the grade of lameness in horse with traumatic arthritis of the fetlock joint.

Key words: rehabilitation, laser therapy, horses, thermal effect, Laser Doppler Flowmetry, histopathology, Met-enkephalin-Arg-Phe, traumatic arthritis, accelerometer technique.

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Papers I-IV

The present thesis is based on the following papers, which will be referred to by their Roman numerals:


Papers I and II are reproduced by permission of the journals concerned.

Abbreviations

CO₂ Carbon dioxide
EIA Enzyme immunoassay
HeNe Helium neon
IR Infrared
LDF Laser Doppler Flowmetry
MEAP Met-enkephalin-Arg-Phe
PGE₂ Prostaglandin E₂
RIA Radioimmunoassay
SP Substance P
Introduction

Physical medicine and rehabilitation

Interest in physical medicine as a method for rehabilitation of animals has grown during the last decades, and so has the need for well designed and validated rehabilitation programs. The primary aim in rehabilitation is to regain best possible physical function after illness or injury. Tissue healing is promoted by stimulation of normal physical processes, thereby restoring the function of injured tissue. To achieve this, the therapist may use different physical modalities.

Rehabilitation generally addresses the consequences of pathology rather than the pathology itself. A “functional diagnosis” is established, based on the impairments present in the animal, i.e. the alterations in anatomical, physiological structures or function as the result of some underlying pathology (International classifications on impairments, disabilities, and handicaps, WHO 2006). Decreased joint ranges of motion, increased muscle tonus and muscle weakness are examples of such impairments. Impairments may lead to a functional limitation, such as inability of the animal to perform.

The design of a rehabilitation protocol should be based on the functional diagnosis, as well as the understanding of the patho-physiological processes of the injured tissues involved. The primary aim of rehabilitation is to restore function and if possible improve healing. In order to facilitate this, different forms of physical treatments have been tried including therapeutic heating. However, there is limited knowledge of the effect of some of the methods and it is imperative to specify the physiological effects and possible side effects of any selected physical modality, since this not only determines the choice of modality - but also when and how to use it.

Rehabilitation using defocused carbon dioxide (CO₂) laser has recently attracted interest (Lindholm et al., 2002) as it has been reported to restore function and improve healing in horses with traumatic arthritis of the fetlock joint. It has been hypothesised that the laser, besides having a specific photobiological effect, induces an increase in local tissue temperature, with a secondary increase in local blood flow. Further, the increase in temperature and blood flow has been suggested to influence pain perception and tissue regeneration (Lehmann, 1990).

Laser light as a rehabilitation tool

The first relatively high power lasers were developed in the 1960s and were used in surgery. During the 1970s, professor Mester in Budapest observed that low levels of laser energy had a “photobiostimulating” effect improving tissue healing in mice skin (Mester et al., 1971). Today, lasers are used in the treatment of both humans and animals. Besides being used as a surgical tool, the main treatment indications are pain relief, wound healing, inflammation and musculoskeletal injuries.
What is a laser?

LASER is the acronym for Light Amplification by Stimulated Emission of Radiation. Laser light is created by devices that convert electromagnetic radiation into narrow-frequency wavelengths of ultraviolet, visible or infrared radiation. A laser device can be characterised according to 1) the medium; i.e. the material which emits excess energy as photons of light with specific wavelengths, 2) the output power that is surgical or high-power (W) or low-level laser (mW), and 3) the potential risk for skin or eye injury; arranged in class I-IV, where class IV involves a definite risk.

The laser device consists of a power source and a chamber with a lasing medium with molecules or atoms that can store and release energy, and with two mirrors at each end (Figure 1).

Laser light is created when energy from the power source excites the atoms or molecules from a ground state to a higher energy level. When returning to the ground state, the excess energy is released as photons. When there is a collision between two stimulated atoms/molecules, both release energy simultaneously in equal amounts, which creates a stimulated emission. Amplification of light is achieved when the photons are reflected back-and-forth between the mirrors. A coherent light is created, which is a highly synchronised light with the waves in phase over long distances. As one of the mirrors is partly reflecting, it transmits a small part of the photons, thus emitting the laser beam. A lasing medium creates light with one specific wavelength, monochromaticity. The infrared (IR) light is classified based on its wavelength into near IR: 760-3000 nm, middle IR: 3000-30000 nm and far IR light: 30000 nm -1mm (Schieke et al., 2003). The classification partly corresponds with the light’s absorption pattern, and for the IR light, the depth of absorption in the skin decreases with increasing wavelength (Figure 2). The light can be emitted continuously or pulsing and scanning devices may be attached to the laser.

Laser types and treatment parameters

When evaluating the effects of laser irradiation a number of physical properties have to be taken into account: wavelength, power, pulse rate, irradiated area, dose at the location of injury, exposure time and treatment intervals.

Laser therapy may be divided into the use of surgical lasers (high-power laser) and lasers used for biomodulation, so-called low-level laser. However, lasers originally made for surgery, such as the CO\textsubscript{2} laser used in the present investigation, are used as biomodulating lasers, with a defocused beam and low output effect.

In low-level laser therapy there is normally an output power ranging between $10^{-3}$ and $10^{-1}$ W, wavelengths between 300 and 10600 nm (Schindl et al., 2000; Nussbaum et al., 2003; Chow & Bamsley, 2005), an energy density between $10^{-2}$ and 1 W/cm\textsuperscript{2}, and a dose of $10^{-2}$ to $10^{2}$ J/cm\textsuperscript{2} (Schindl et al., 2000). A high-power laser has a power range of $10^{-3}$ W to hundreds of W (Carruth & McKenzie, 1986). The laser beam is usually focused when used as a surgical laser, and the dose can be several hundreds to thousands J/cm\textsuperscript{2} (see Table 1).
The laser/tissue interaction

Laser light may be reflected, transmitted, absorbed or scattered (change of direction). The depth of penetration is defined as the depth where the intensity of the light is approximately 36% of the original intensity (Baxter, 1994). It is difficult to predict the exact amount of absorption and scattering because of the non-homogenous properties of tissue. In general, the degree of scattering decreases with increasing wavelength. Consequently, the tissue reaction is dependent on the wavelength, as the wavelength of a laser beam determines the absorption and the depth of penetration (Hecht, 1992).

Fig. 1. Simplified design of a CO$_2$ laser.

Fig. 2. Wavelengths of two lasers and their relative absorption depth in skin.
UV = ultraviolet light,
IR = infrared light.
HeNe = helium-neon laser,
CO$_2$ = carbon dioxide laser.
If absorbed, light energy is converted into other forms of energy such as thermal or chemical energy. Simplified, visible and near ultraviolet light are absorbed by chromophores (light-sensitive molecular structures), such as haemoglobin and melanin. Infrared light is absorbed by water and can induce vibrational changes in biomolecules (Hecht, 1992). Thus, the transmission of light with 1200 nm and longer wavelengths through epidermis is dependent on the thickness and water content but not on the pigmentation of epidermis (Andersson, 1994).

Table 1. Dosimetric parameters of laser irradiation (after Schindl, 2000)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Comments</th>
<th>Units</th>
<th>Low level</th>
<th>High-power</th>
</tr>
</thead>
<tbody>
<tr>
<td>Radiant flux (Power output)</td>
<td>Power = energy/time (W=J/s)</td>
<td>W</td>
<td>$10^{-3}$-1</td>
<td>$&gt;10^1$</td>
</tr>
<tr>
<td>Irradiance (Power density, Intensity)</td>
<td>Irradiance = power/area irradiated</td>
<td>W/cm²</td>
<td>$10^{-2}$-1</td>
<td>&gt;1</td>
</tr>
<tr>
<td>Dose (Energy density, Radiant exposure)</td>
<td>Dose = power X irradiation time/area</td>
<td>J/cm²</td>
<td>$10^{-2}$-$10^2$</td>
<td>$&gt;10^2$</td>
</tr>
<tr>
<td>Pulse rate</td>
<td>Pulses/ second</td>
<td>Hz</td>
<td>0- 5000</td>
<td></td>
</tr>
<tr>
<td>Pulse duration</td>
<td>Time when laser light is emitted</td>
<td>ms</td>
<td>1-500</td>
<td></td>
</tr>
<tr>
<td>Pulse interval</td>
<td>Time when the laser is off between pulses</td>
<td>ms</td>
<td>1-500</td>
<td></td>
</tr>
<tr>
<td>Treatment time</td>
<td>Total time when the laser light is emitted</td>
<td>s</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Possible mechanisms of action

Despite of the frequent use of laser therapy there is a lack of knowledge about its mechanisms. Effects such as local circulation enhancement (Kubota, 2002), anti-inflammatory effect (Campana et al., 1999; Campana et al., 2004; Bjordal et al., 2006), enhancement of cartilage and bone healing (Chen & Zhou, 1989; Tsai et al., 1997; Cho et al., 2004), and peripheral nerve stimulation and analgesic effect (Wesselmann et al., 1991; Baxter et al., 1992) have been suggested. Although several biological mechanisms have been proposed to explain this wide range of effects, the data available is difficult to interpret owing to a wide diversity in experimental protocols and studies (Basford, 1995; Bjordal et al., 2003; Chow & Barnsley, 2005).

The photobiological effects of laser irradiation have been attributed to photochemical, photothermal and photomechanical changes (Nussbaum et al., 2003). The photochemical effect has been suggested to caused by excitation of light-sensitive molecules (photoacceptors, chromophores), some of which are
suggested to be cytochrome enzymes in mitochondria and cell membranes (Karu, 1989). Photothermal effects result from transformation of absorbed light energy to heat (Thomsen, 1991; Hecht, 1992). Photothermal effects are unlikely to apply to low-level laser therapy, as temperatures in skin are elevated by less than 1 °C (Stadler et al., 2004). Photomechanical effects are secondary to rapid heating with ultrashort laser pulses, causing cell and tissue damage.

The defocused CO$_2$ laser

The defocused CO$_2$ laser is a high-power laser, emitting infrared light in the far IR spectrum, Table 2.

Table 2. Lasing medium parameters (KSV 25S Laser device)

<table>
<thead>
<tr>
<th></th>
<th>CO$_2$</th>
<th>HeNe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wavelength</td>
<td>10600 nm</td>
<td>633 nm</td>
</tr>
<tr>
<td>Continuous output power</td>
<td>0-25 W</td>
<td>1.2 mW</td>
</tr>
<tr>
<td>Mode of application</td>
<td>scanning</td>
<td>scanning</td>
</tr>
<tr>
<td>Diameter of beam at source</td>
<td>6 mm</td>
<td>0.61 mm</td>
</tr>
<tr>
<td>Divergence</td>
<td>1.5 mrad</td>
<td>2.0 mrad</td>
</tr>
</tbody>
</table>

In the present studies, light was continuously emitted through a scanning device causing a quasi-continuous delivery of radiation with a 6 mm diameter large laser spot scanning back-and-forth over the treatment area.

Since soft tissue consists of 70-80% water (human skin), it is assumed that the tissue absorbs infrared light as it would do in water (Hecht, 1992). The penetration depth of CO$_2$ laser is about 20 μm in water (Hecht, 1992). Absorption in tissue is approximately 90% within the first 100 μm (Carruth & McKenzie, 1986) and scattering is negligible (Thomsen, 1991). The long wavelength and absorption pattern of the CO$_2$ laser produce direct kinetic excitation rather than electronic excitation and resulting in a photothermal effect (Anderson, 1994). The CO$_2$ laser is a potent laser device, with a thermal effect ranging from very mild warming to coagulation and carbonation of tissues (Thomsen, 1991). Incorrect use of the CO$_2$ laser may therefore cause serious side effects.

Traumatic arthritis

Fetlock arthritis is a commonly registered diagnosis in Swedish insured riding and leisure horses (Penell et al., 2005). The inflammation causes distress to the horse and limits its ability to normal function. Most lesions are induced by acute trauma, repetitive loading or overload (Howard & McIlwraith, 1996).
**Symptoms**

Traumatic arthritis is often accompanied by synovitis, with symptoms of synovia effusion, increased skin temperature over the joint area, a palpable thickening of the joint capsule, a decrease in joint range of motion and lameness. Cartilage damage may also be present.

There is a significant relationship between synovitis, capsulitis and articular cartilage damage. The local responses are influenced by inflammatory mediators such as prostaglandin \( E_2 \) (PGE\(_2\)) and substance \( P \) (SP), which can function as indicators of the severity of synovitis (Bertone, 2001). Besides causing vasodilatation, SP and PGE\(_2\) influence the threshold in mechanoreceptors (Birrell et al., 1991; Schaible, 2006) and pain may be elicited by mechanical stimuli such as palpation, movement within normal range of motion, and weight loading, which normally would not elicit pain (Schaible, 2006).

**Neurogenic inflammation**

Recent studies have demonstrated that the peripheral nerves contribute to the inflammatory response, so-called neurogenic inflammation, by the release of inflammatory substances (Löfgren et al., 1997). One of these substances is substance \( P \) (SP), a member of the tachykinin family of neuropeptides. It is detected in small diameter unmyelinated nerves of periarticular periosteum, joint capsule, ligaments and subintimal synovia layers of horses (Nixon & Cummings, 1994). Down-regulation of the inflammatory response is mediated by the release of opioids from immuno-cells in the inflammatory tissue. The opioid peptide Met-enkephalin-Arg-Phe (MEAP) is an important neuromodulator in anti-nociception and inflammation (Rosen et al., 2000). According to Caron et al. (1996), the nociceptive articular nerves have two objectives - they transmit the pain signal to the CNS, and when activated they release neurotransmitters into the synovial membrane and fluid thus modulating inflammation.

**Diagnosis and treatment**

The diagnosis is traditionally made by conventional lameness examination: grade 0-5 (Åsheim & Lindblad, 1976), palpation, radiographs and analysis of blood and synovia. Flexion tests and intra-articular analgesia may help to locate the site of pain.

The conventional treatment of traumatic arthritis focuses on pain relief and functional recovery by inhibiting cartilage deformity factors as much as possible. The treatment of choice is often intra-articular injections with corticosteroids and/or sodium hyaluronate, alone or together with systemic non-steroid anti-inflammatory drugs. Current treatment includes complementary methods, of which laser therapy is one modality used.

To my knowledge, only one study has described the clinical outcome of defocused CO\(_2\) laser therapy in horses (Lindholm et al., 2002). It reports a decrease in lameness after treatment of traumatic fetlock arthritis (10600 nm, 60 J/cm\(^2\)) superior to the effect of intra-articular injection of betamethasone in combination with sodium hyaluronate.
A comparison of treatment protocols in humans is often difficult due to inadequate specifications of treatment parameters (Beckerman et al., 1992; Basford, 1995; Bjordal et al., 2003; Chow & Barnsley, 2005) and direct extrapolation of benefits from humans to horses is difficult to make (Ramey & Basford, 2000).

Assessment of treatment outcome

Accelerometer technique

The main measure of a successful treatment of musculoskeletal injuries is the reduction of pain, frequently evaluated as a reduction in lameness by conventional lameness examination. However, an accelerometer technique has been suggested to be a more objective method (Weishaupt et al., 2001; Keegan et al., 2002; Leleu et al., 2004) and the technique allows detection of subtle changes in the movement pattern of horses.

Aims of the investigation

The overall hypothesis of this investigation was that treatment with defocused CO₂ laser causes a reduction in the degree of lameness. The principal aim was to evaluate the effects of defocused CO₂ laser on equine tissues, using objective methods also applicable to other rehabilitation modalities.

The specific aims of the investigation were to:

- measure the effect of defocused CO₂ laser on local temperature and blood flow in equine tissue by use of temperature probes and Laser Doppler Flowmetry technique
- investigate the effects of different dosages of defocused CO₂ laser on equine skin morphology by light microscopy
- determine the concentration of inflammatory mediators and opioids in synovia, i.e. substance P, PGE₂ and Met-enkephalin-Arg-Phe
- examine the effect of defocused CO₂ laser treatment on traumatic inflammation of the fetlock joint, evaluated by conventional lameness examination and accelerometer technique.

Materials and methods

Materials and methods are described in detail in the papers I-IV, whereas a more general description will be presented herein. The research plan and procedures involving the use of animals were reviewed and approved by the Ethical Committee on Animal Experiments in Uppsala, Sweden.
Horses

The horses in Study I-III were all healthy Standardbred trotters owned by the Department of Large Animal Sciences, SLU, Uppsala, Sweden. Study IV comprised 16 privately owned horses of various breeds, from a russ pony to Swedish Warmblood horses. They were all referred to the University equine clinic due to forelimb lameness. The horses were examined according to the research protocol and if the horse met all inclusion criteria, a written consent allowing the horse to be included in the study was obtained by the owner. Table 3 summarises data for the horses investigated in the respective studies.

Table 3. Summarised data for the horses investigated in the present studies

<table>
<thead>
<tr>
<th>Group</th>
<th>No.</th>
<th>Breed</th>
<th>Age (year)</th>
<th>Weight (kg)</th>
<th>Sex</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>10</td>
<td>Stb.Tr.</td>
<td>7 (3-13)</td>
<td>489 (403-580)</td>
<td>4 mares, 6 geldings</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>Stb.Tr.</td>
<td>7 (2-20)</td>
<td>501 (375-580)</td>
<td>1 gelding</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>Stb.Tr.</td>
<td>9 (2-24)</td>
<td>464 (375-548)</td>
<td>4 mares, 3 geldings</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>Stb.Tr.</td>
<td>8 (5-13)</td>
<td>483 (460-507)</td>
<td>4 mares, 2 geldings</td>
</tr>
<tr>
<td>II</td>
<td>10</td>
<td>Stb.Tr.</td>
<td>9 (4-19)</td>
<td>497 (411-578)</td>
<td>6 mares, 4 geldings</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>Stb.Tr.</td>
<td>10 (2-24)</td>
<td>486 (375-578)</td>
<td>8 mares, 5 geldings</td>
</tr>
<tr>
<td>IV</td>
<td>16</td>
<td>Various*</td>
<td>9 (4-18)</td>
<td>not registered</td>
<td>5 mares, 11 geldings</td>
</tr>
</tbody>
</table>

Age and weight are given as the group median with the range within parenthesis. No = numbers; Stb.Tr. = Standardbred trotter; Anaest = anaesthetised horses. *See study IV for a specification of the different breeds of horses.

Procedures

Local tissue temperature and blood flow, skin morphology, laboratory analysis of blood and synovia and lameness examination were used to assess possible physiological and clinical effects of defocused CO₂ laser. Blood flow measurements and skin biopsies were performed during anaesthesia, as well as some of the temperature recordings and blood and synovia samplings. Table 4 shows an overview of the different procedures used in each study. In depth details on each study design are given in paper I-IV.

Temperature measurements in Study I and II were performed on the skin and subcutis of the dorsal side of the fetlock, and in the fetlock joint. An area of 6 x 7 cm on the lateral, dorsal and medial sides of the fetlock was clipped. The lateral side was washed with antiseptic solutions (Hibiscrub, Hibitan; Zeneca, Göteborg, Sweden). Joint temperature: a flexible probe, 0.8-mm diameter (MAA-08500-A; ELLAB, Rådovre, Denmark), was inserted 3–4 cm into the joint, from the lateral aspect. Subcutis temperature: a needle probe measuring 0.8 mm in diameter
(MKA-08050-A; ELLAB, Rødovre, Denmark) was inserted approximately 15 mm under the skin, in the midline of the dorsal side of the fetlock joint and 2 cm proximal to the treatment area. Skin temperature: a skin probe (MHB-08025-A; ELLAB, Rødovre, Denmark) was attached with adhesive tape over the midline of the dorsal side of the fetlock, 2 cm proximal to the treatment area. After each treatment occasion, the probes were controlled in a water bath using a mercury thermometer.

In Study I, the effect of defocused CO\textsubscript{2} laser on the temperature of the skin, subcutis and fetlock joint was examined in standing and anaesthetised horses.

The experiment on the standing horses was a cross-over study with randomised laser and sham irradiation (fictitious irradiation, i.e. the laser beam directed on the non-reflecting floor). Temperature probes were attached to the skin and subcutis on the dorsal side of the fetlock, and inserted into the joint as illustrated by Figure 3. In two horses, the location of the temperature probe in the joint was confirmed by radiographs. Consecutive irradiations of 91 J/cm\textsuperscript{2} were applied to the lateral, dorsal and medial aspects for 4 min, respectively. Temperature was measured every 30 s from 5 min before the start of the irradiation, during the irradiation, and 5 min after irradiation.

Three studies were conducted on anaesthetised horses in order to avoid movements of the temperature probes and to obtain data over a longer period. The first study comprised 12 horses; eight received laser irradiation and four served as controls. The horses were anaesthetised, placed in left recumbency and irradiated with 137 J/cm\textsuperscript{2} (16 W, 6 x 7 cm) on the dorsal aspect of the fetlock joint. Temperatures in skin, subcutis and fetlock joint were measured every 30 s from 5 min before the start of the irradiation, during the 6 min of irradiation and until 30 min after irradiation (recordings were carried out every min from 10 min after the end of treatment).

A second cross-over experiment was carried out on seven anaesthetised horses in order to examine changes in temperature between clipped and unclipped hair coat. A treatment area (6 x 7 cm) on each side of the croup was prepared; one side clipped and the other side with the hair coat intact. Skin and subcutis temperature probes were attached and irradiation of 171 J/cm\textsuperscript{2} (20 W) was carried out randomly. The temperature recordings were done every 30 s from 5 min before the start of the irradiation, during the 6 min of irradiation, and 5 min after irradiation.
Table 4. An overview of different procedures of each study

<table>
<thead>
<tr>
<th>Study</th>
<th>Group</th>
<th>Premed</th>
<th>Induction</th>
<th>Anaesthesia</th>
<th>Laser protocol</th>
<th>Irradiation area</th>
<th>Assessment</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Standing</td>
<td>Ace</td>
<td>GG+Thio</td>
<td>Halothane</td>
<td>91 J/cm²</td>
<td>Fetlock</td>
<td>Temperature, blood and synovia</td>
</tr>
<tr>
<td></td>
<td>Anaesthesia</td>
<td>Meth</td>
<td></td>
<td></td>
<td>137 J/cm²</td>
<td>Fetlock</td>
<td>Temperature, blood and synovia</td>
</tr>
<tr>
<td></td>
<td>Anaesthesia</td>
<td>Det</td>
<td>GG+Thio</td>
<td>Isoflurane</td>
<td>171 J/cm²</td>
<td>Croup</td>
<td>Temperature, blood and synovia</td>
</tr>
<tr>
<td>II</td>
<td>Anaesthesia</td>
<td>Det</td>
<td>GG+Thio</td>
<td>Isoflurane</td>
<td>91 J/cm²</td>
<td>Fetlock</td>
<td>Temperature, blood and synovia</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>137 J/cm²</td>
<td>Fetlock</td>
<td>Temperature, blood and synovia</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>171 J/cm²</td>
<td>Croup</td>
<td>Temperature, blood and synovia</td>
</tr>
<tr>
<td>III</td>
<td>Anaesthesia</td>
<td>Ace</td>
<td>GG+Thio</td>
<td>Halothane</td>
<td>91 J/cm²</td>
<td>Hamstring</td>
<td>Temperature, blood flow</td>
</tr>
<tr>
<td></td>
<td>Anaesthesia</td>
<td>Meth</td>
<td></td>
<td></td>
<td>137 J/cm²</td>
<td>Fetlock</td>
<td>Skin biopsy</td>
</tr>
<tr>
<td></td>
<td>Anaesthesia</td>
<td>Det</td>
<td>GG+Thio</td>
<td>Isoflurane</td>
<td>450 J/cm²</td>
<td>Loin</td>
<td>Skin biopsy</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>91 J/cm²</td>
<td>Hamstring</td>
<td>Skin biopsy</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>137 J/cm²</td>
<td>Fetlock</td>
<td>Skin biopsy</td>
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<td>450 J/cm²</td>
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<td>Skin biopsy</td>
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<td>Standing</td>
<td></td>
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<td>91 J/cm²</td>
<td>Fetlock</td>
<td>Lameness evaluation, blood and synovia samples</td>
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<td></td>
<td>Traumatic arthritis</td>
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</table>

Abbreviations: Premed = premedication; Ace = acepromazine; GG = guaifenesin; Thio = thiopentone; Meth = methadone; Det = detomidine.
During the course of the previous experiments we detected that the joint temperature fluctuated before and during irradiation. Therefore, a third extended experiment was carried out on six anaesthetised horses; four received laser and two served as controls, in order to await a steady state in temperature before the start of laser irradiation. A temperature probe was inserted into the fetlock joint and after a steady state in temperature was reached, the dorsal side of the fetlock area was irradiated with 91 J/cm² (16 W, 4 min, 6 x 7 cm). Temperature was registered every minute until a new steady state was reached after irradiation.

In all studies, blood samples from the jugular vein and synovia samples from the fetlock joint were collected, before and after each irradiation of the joint area.

The aim of Study II was to examine if an increase of the temperature of equine superficial tissue during laser irradiation was accompanied by an increase in the local blood flow. Ten horses were anaesthetised and placed in dorsal recumbency. A treatment area of 6 x 7 cm was prepared on each semimembranosus muscle; one side clipped and the other with the hair coat intact. A small area for the measuring probes was prepared in direct contact with each irradiated area. Skin temperature and perfusion were measured on the skin surface (for further details on Laser Doppler Flowmetry, see page 18), 1 and 3 cm from the irradiated area. Muscle temperature and perfusion probes were inserted to a depth of 3 cm, approximately 1 cm from its corresponding 1 cm skin probe, and at the same distance from the irradiated area. The treatment areas were randomly irradiated with 91 J/cm² or with sham laser (with the beam directed away from the horse). The temperatures were displayed and recorded continuously (Perisoft 1; 14, Perimed, Järfälla, Sweden) for approximately 50 min. The average values during one minute before irradiation, during irradiation, the peak value during irradiation and the time to the peak value were analysed. Blood samples from the jugular vein and synovia fluid from the fetlock joint were taken before and after irradiation.

In Study III, the aim was to describe in vivo effects of three different doses of defocused CO₂ laser on equine skin morphology. Thirteen of the horses from Study I and II were irradiated on three sites. Treatment areas were clipped and irradiation over the proximal semimembranosus muscle (hamstring: 91 J/cm²; 16 W, 6 x 7 cm, 4 min), on the dorsal fetlock (137 J/cm²; 16 W, 6 x 7 cm, 6 min), and on the loin (450 J/cm²; 20 W, 4 x 4 cm, 6 min) was conducted. Approximately 90 min after irradiation, skin biopsies were taken from irradiated and corresponding non-irradiated sites (for Skin biopsy technique, see page 19). The horses that received the 137 and 450 J/cm² doses were kept anaesthetised since they were designated to be used for surgical training and were subsequently euthanised with an overdose of pentobarbitone sodium (Avlivningsvätska för djur, 100 mg/ml, Apoteksbolaget AB, Umeå, Sweden).

In Study IV, the effect of defocused CO₂ laser on traumatic inflammation of the fetlock joint was evaluated by conventional lameness examination and an objective accelerometer technique (for Accelerometer technique, see page 21), as well as analyses of substance P, PGE₂ and Met-enkephalin-Arg-Phe in synovia (for Blood and synovia analyses, see page 20). The inclusion criteria for the study were: the horse should be used for riding/trotting and be more than 3 years of age,
have an initial lameness graded between 0.5 and 2 degrees in a forelimb, demonstrate a decrease (≥ 75%) in lameness after intra-articular anaesthesia of the fetlock joint, and have normal to minor findings on radiographs of the actual joint.

The horses stayed in the clinic for one week and were walked by hand for 2 x 10 min daily. Eight horses received laser treatment and eight received sham treatment in a random order. Blood samples from the jugular vein and synovia from the fetlock joint were collected 1 day before the treatment started. Consecutive irradiations of 91 J/cm² (16 W, 6 x 7 cm) were applied to the lateral, dorsal and medial aspects of the fetlock for 4 min, respectively, on five occasions during one week (once daily, with a non-treatment day after the first session). For the horses that received sham treatment, the laser beam was directed away from the horse. The horses were re-examined by the same blinded clinician using the same protocol at 7 and 21 days after the initial examination and blood and synovia samples were withdrawn.

Techniques

Laser Doppler Flowmetry

Laser Doppler Flowmetry (LDF) is a completely different use of laser light. It is a technique designed to provide continuous measurements of microvascular perfusion, in terms of relative changes of blood volume and velocity over time at a single site (Nilsson et al., 1980; Öberg et al., 1984). When the tissue is illuminated by the LDF laser light (780 nm) from a fibre-optic probe, the light photons hit both moving red blood cells and static structures. The light that hits moving structures changes its direction causing a shift of the Doppler frequency, which is directly related to the number and velocity of the blood cells. The scattered light is then re-emitted from the tissue and the wavelength shift is captured by a photodetector that produces a LDF signal. The instrument measures blood flow in a tissue volume of about 1-1.5 mm³ and is integrated over the entire volume measured. Thus, the units are arbitrary and reflect relative changes in perfusion. The unknown orientation of the vessels leads to a variability in the perfusion measured in different subjects and in the same subject, if the probe is repositioned.

To allow comparison of results, a calibration of the probes is made in a standard motility solution provided by the manufacturer.

In Study II, skin and muscle blood perfusion was measured by LDF, using a Periflux 4001 flowmeter with additional microtips and probes (Perimed, Järfälla, Sweden). A small area for the measuring probes was surgically prepared, in direct contact with the treatment area over the proximal semimembranosus muscle. Skin perfusion was measured on the surface (Probe 407), 1 and 3 cm from the irradiated area. For muscle perfusion, a straight microtip with slanted tip (MT A500-0.120 mm, 0.5 mm diameter) was inserted into the semimembranosus muscle of the right and left hind limb, close to the skin perfusion probe at 1 cm from each irradiated area (Figure 4).
The microtip was inserted via a 0.7 mm cannula to a depth of 3 cm and the cannula was retracted. Thereafter, the microtip was connected to a probe (Master Probe; Probe 418-x, Perimed, Järfälla, Sweden). Blood flow (flux), expressed in blood perfusion units (PU), was displayed and recorded continuously. The data were transferred to a computer using a software program (Perisoft 1; 14, Perimed, Järfälla, Sweden). The average values during one minute before irradiation, during irradiation, the peak value during irradiation and the time to the peak value were analysed.

**Skin biopsy technique**

Skin samples in Study III were obtained from the dorsal aspect of the fetlock, the loin and the proximal area of semimembranous muscle (hamstring) with an 8 mm disposable biopsy punch (Miltex Instrument Company, Inc.). The samples were taken from the centre of the laser-irradiated and corresponding non-irradiated control sites, approximately 90 minutes after the laser irradiation. The skin samples were divided into two parts along the longitudinal axis, and were immediately fixed in cacodylate-buffered 3% glutaraldehyde (pH 7.2). The specimens were rinsed in 0.067 M cacodylate buffer (pH 7.2), dehydrated in a graded series of ethanol, infiltrated and embedded in Historesin (Leica Microsystems Nussloch GmbH, Heidelberg, Germany). Sections of 2μm thickness were cut and stained with haematoxylin-eosin (H&E). The sections were examined with special reference to the appearance of epidermis, epidermis-dermis junction, adnexal structures, cell infiltrates and blood vessel appearance in the dermis. Furthermore, computer-assisted measurements of epidermal thickness (Easy Image Analysis System) were obtained on every fiftieth micrometer of all sections and the shortest distance between the dermal-epidermal junction and the innermost aspect of the stratum corneum were measured.
Blood and synovia analyses

Blood samples in Study I, II and IV were collected from the jugular vein and synovia samples (after surgical preparation) from the lateral aspect of the fetlock joint, before and after laser irradiation. Vials containing EDTA were used for measurement of white blood cell count and serum vials were used for determination of total plasma protein concentration according to the routine methods used at the Division of Clinical Chemistry, SLU. Additional synovia samples were centrifuged (2 x 20 min, 600 x g) and stored at -80 °C until being analysed.

Measurements of substance P (SP) and Met-enkephalin-Arg-Phe (MEAP)

The synovial fluid samples (230 µl) were acidified by adding 200 µl of 0.1 M HCl and homogenised by ultrasonication. The homogenates were diluted (1:10) with 0.1 M formic acid /0.018 M pyridine, pH 3.0 (buffer I) and subsequently centrifuged for 2 min at 3000 x g (4 °C). The supernatant fractions were purified by ion exchange chromatography. Small plastic columns were packed with SP-Sephadex C-25 gel (GE Healthcare, Uppsala, Sweden, packed gel volume = 1 ml) and washed with 20 ml buffer I before the samples were added. After additional washing with 10 ml buffer of I and 5 ml of 0.1 M formic acid /0.01 M pyridine (buffer II), the fraction containing SP and MEAP was eluted with 4 ml of 1.6 M formic acid /1.6 M pyridine, pH 4.4 (buffer V). All buffers (I, II, V) contained 0.01% mercaptoethanol. The eluates were evaporated in a Speed Vac centrifuge (Savant, Hicksville, NY, U.S.A.) and subsequently analysed by radioimmunoassay (RIA).

The radioimmunoassay for SP and MEAP were based on the charcoal adsorption technique and were conducted as described by Hallberg et al. (2000) and Johansson et al. (2000). For all RIAs the antibodies were raised in rabbits against the peptide-thyroglobulin conjugate and 125I-labelled Met-enkephalin-Arg⁴-Phe⁷ or Tyr⁵-substance P were used as tracers. The cross-reactivity for the antiserum with SP fragments (3-11), (5-11) and (6-11) was 100, 60 and 20% respectively, with all SP N-terminal fragments and other SP related peptides less than 0.1% and the detection limit of the RIA was about 5 fmol/tube (Sharma et al., 1990; Hallberg et al., 2000). The cross-reactivity of the MEAP antiserum was 0.5% with Met-enkephalin-sulphoxide and less than 0.1% with Met-enkephalin, Met-enkephalin-Lys⁶, Leu-enkephalin-Arg⁶ and Leu-enkephalin (Johansson et al., 2000). The intra-assay variance was 8.5% for SP and 40% for MEAP.

Measurements of PGE₂

The concentration of PGE₂ was determined by direct enzyme immunoassay (EIA) as previously described by Skarzynski et al. (2000). Cross-reactivity for the antiserum was: PGE₂ 100%; PGE₁ 18%; PGA₁ 10%; PGA₂ 4.6%; PGB₁ 6.7%; PGD₂ 0.13%; PGF₂α 2.8%; PGJ₂ 14% and 15-keto PGE₂ 0.05%. The PGE₂ standard curve ranged from 0.08 ng/ml to 20 ng/ml. The intra-assay coefficient of variation was 8.4%.
**Accelerometer technique**

Accelerometer technique is a method to use signal processing to analyse data from accelerometers. Two two-axis differential-capacitance accelerometers (ADXL250, Analog Devices, USA) were mounted perpendicular to each other on a board together with analogue components, memory, a/d-converters, battery and RF-equipment, which altogether were mounted in a box. The accelerometers measured acceleration, ±50 g, with a resolution of 0.01 g and a sampling frequency of 1000 Hz in three directions; x, y and z, thus creating three-dimensional acceleration data. The accelerometers measure the instant change of velocity during a given interval that corresponds to the acceleration applied at the position of the accelerometer. The acquisition duration was 10 s. Data were sampled synchronously from all seven transducers with software developed by Rolab AB (Glunten, Uppsala, Sweden). Data sampling was triggered telemetrically and after the recordings a number of data recordings were downloaded from the transducers devices to a PC via a serial interface for further analysis.

The conventional lameness examination in Study IV was complemented with the accelerometer technique. The transducers were fixed to pockets in boots, girths and a neck piece. The seven transducers were applied to the four legs, wither, neck and croup (Figure 5), with approximately horizontal and vertical measuring axes, and an offset set to zero. Three separate accelerometer recordings of the initial lameness were made at slow trot by hand. Thereafter a flexion test of the phalanges was performed followed by new recordings. An intra-articular analgesia was performed to secure the diagnosis, and accelerometer recordings were carried out again before and after a second flexion test.

In order to get more detailed data for a calculation of the correlation between the subjective (conventional) and objective (accelerometer) lameness grading, a visual re-evaluation was done from the video-recordings by the same clinician (blinded). The re-evaluation was performed randomly on all horses and registrations, after completing the practical part of the study.

**Accelerometer data analysis**

Data from the dorso-ventral and longitudinal axis of the transducer on the withers and the dorso-ventral axis of the transducer on the neck were selected for further analysis. Power spectrum for the three-time series and subsequently the quotient between the first and second harmonic were calculated. Data from each recording of the initial lameness were averaged.

The second harmonic (at approximately 2.5 Hz) represents the step frequency, which in trot is half of the stride frequency. If the trot is symmetrical this will be the dominant harmonic. In case the horse is lame, i.e. asymmetrical, a harmonic (the first harmonic) equal to the stride frequency will start to increase. Consequently, the quotient between the first and second harmonic will constitute an objective measure of the gait asymmetry in trot. The three quotients were summed and used as an objective parameter of the gait asymmetry.
**Fig. 5.** Accelerometer devices adapted to the neck, withers, croup and legs.

### Statistical analysis

Statistica software (Statsoft Inc., Tulsa, OK, USA) was used for statistical analyses in all articles.

Before the statistical calculations in Study I, the data were individually corrected by subtracting the mean for each individual pre-treatment period. The mean temperature was calculated from the recordings performed every 30 s during each time period: pre-treatment, treatment and post-treatment. Thereafter, the area under the curve was calculated in order to describe the total heating effect during a specific time period.

Before the statistical calculations in Study II, perfusion data were individually corrected by setting the baseline before treatment to 100% and treatment data were compared to baseline data within each group. Perfusion values less than 3.5 perfusion units (PU) were excluded from the analysis as the mean value for biological zero (i.e. the LDF signal from non-perfused tissue) for equines is approximately 1.6 PU for skin and 3.5 PU for muscle (Edner, 2005).

In Studies I and II, temperatures and perfusions were compared using non-parametric tests. A Wilcoxon test was applied to detect within-group differences and a Mann-Whitney U test for differences between groups.

In Study III, epidermal thickness and changes in skin morphology were compared by Student’s paired t-test for within-group differences and un-paired t-test for differences between groups.

In Study IV the correlation between the accelerometer data and conventional lameness score was performed with a Spearman’s rank correlation test. All changes in lameness and in blood and synovia parameters were determined by calculating the data before and after treatment for each horse, comparing the differences within each horse with a Wilcoxon test and between groups with a
Mann-Whitney U test. In all studies, statistical significance was accepted at p<0.05.

Results

Temperature in skin, subcutis and the fetlock joint
(Study I and II)

The temperature results are demonstrated by Figure 6 and summarised in Table 5. The increase in the temperature of skin and subcutis in Study I was significantly higher during laser irradiation compared to the controls in both standing and anaesthetised horses. In standing horses, the average increase in skin measured during irradiation from the dorsal side was 5.3±1.4 °C and 0.5±0.5 °C for laser irradiated (91 J/cm²) and control, respectively. The increase in subcutis was 5.7±1.0 °C and 1.8±0.7 °C. No significant difference in the increase in temperature was observed between the irradiated and control fetlock joints (1.8±0.4 °C and 2.9±0.7 °C, respectively).

![Figure 6](image)

**Fig. 6.** Overview of temperature curves in a) skin and b) subcutis for standing horses.

*Significantly different from control group.

The results were similar in the anaesthetised horses. The average increase measured during irradiation from the dorsal side, was 3.2±0.7 °C and 0.0±0.2 °C in skin, and 5.5±1.4 °C and -0.2±0.4 °C in subcutis, for the laser irradiated (137 J/cm²) and controls, respectively. A significant difference in skin temperature...
was found during the 5 min period after laser irradiation compared to the controls (2.2±0.5 °C and 0.3±0.0 °C, respectively).
A significant difference in temperature of subcutis was seen both during the first 5 min period (2.9±0.8 °C and 0.1±0.3 °C, respectively) and the following 5 min period (1.7±0.5 °C and 0.3±0.1 °C, respectively) after laser irradiation compared to the controls.
The temperature in the fetlock joint was not altered by laser irradiation.
A significant difference in skin temperature between clipped and unclipped areas (5.2±1.4 °C and 11.3±2.6 °C, respectively) was observed after laser irradiation with a dose of 171 J/cm², Figure 7.

![Fig. 7. Overview of temperature curves in a) skin and b) subcutis for anaesthetised horses. The curves to the right demonstrate the differences between unclipped and clipped hair coat, respectively. *Significantly different from control group.](image)

In Study II, there was a significant increase in temperature in all skin recordings, i.e. 1 cm and 3 cm from the irradiated area, compared to the respective baseline recordings, for both clipped and unclipped hair coat (1 cm clipped 5.5±1.5 °C and unclipped 4.8±1.4 °C, 3 cm clipped 5.5±1.5 °C and unclipped 2.1±0.4 °C). No significant difference in muscle temperature measured 1 cm from the irradiated area and at the depth of 3 cm, was observed.
Table 5. An overview of temperature response to defocused CO2 laser irradiation

<table>
<thead>
<tr>
<th></th>
<th>Dose (J/cm²)</th>
<th>Pre-treatment (°C)</th>
<th>Treatment (°C)</th>
<th>Post-treatment (°C)</th>
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<tr>
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<td></td>
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<td></td>
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<tr>
<td>Laser</td>
<td>91</td>
<td>29.5±1.6</td>
<td>34.8±1.5*</td>
<td>31.9±1.1</td>
</tr>
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<td>91</td>
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<td>30.3±1.5</td>
<td>30.5±1.6</td>
</tr>
<tr>
<td>Subcutis (n=10)</td>
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<tr>
<td>Laser</td>
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<td>36.0±0.9*</td>
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<td>31.9±0.9</td>
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<tr>
<td>Fetlock joint (n=9)</td>
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<td></td>
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<tr>
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<td>33.3±1.0</td>
<td>35.1±0.7</td>
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<td>30.2±0.4</td>
<td>33.5±0.9*</td>
<td>32.3±0.5*</td>
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<td>37.4±1.4*</td>
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<tr>
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<td>Fetlock joint (n=12)</td>
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<td>33.4±0.6</td>
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<td>137</td>
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<td>Subcutis (n=6)</td>
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<tr>
<td>Unclipped</td>
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<td>37.2±1.0</td>
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<td><strong>Extended study</strong></td>
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<td>29.9±0.7</td>
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<tr>
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<td>29.4±0.4</td>
<td>29.4±0.4</td>
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</tr>
</tbody>
</table>

Data is presented as mean±standard error (SE). The pre- and post-treatment recordings were performed during 5 min. *Significantly different from control group, # significantly different from unclipped group (p<0.05).

**Blood flow in skin and muscle (Study II)**

As demonstrated by the laser recording in Study II, the increase in temperature was immediately followed by an increase in perfusion (Figure 8). A significant increase in perfusion was found in all skin recordings, i.e. 1 and 3 cm from the irradiated area for both clipped and unclipped hair coat (1 cm clipped 334.0±170.5%, unclipped 263.8±120.2%; 3 cm clipped 245.2±123.6%, unclipped 116.5±65.3% compared to baseline measurements set to 100%). There was no significant difference in muscle perfusion in either laser irradiated or control horses. Nor was there a significant difference in the peak value or time to peak for skin.
Fig. 8. Representative tracing from one laser treated and one control horse, displaying temperature and blood perfusion response in skin and semimembranosus muscle. The perfusion is presented as arbitrary Perfusion Units (PU) and temperature as °C.

Channel 1; perfusion in muscle.
Channel 2; perfusion in skin at 1 cm.
Channel 3; perfusion in skin at 3 cm.
Channel 4; temperature in muscle.
Channel 5; temperature in skin 1 cm.
Channel 6; temperature in skin at 3 cm.

A = one minute tracing immediately before the start of the treatment.
B = one minute tracing at the end of the treatment.
C = one minute tracing at four minutes after end of the treatment.
T = start of laser and sham laser treatment, respectively.

Skin morphology (Study III)

No macroscopic changes were detected, except erythema, observed at the loin approximately 5-10 min after irradiation.

Four different categories of morphological changes were observed (for illustrations, see Study III). The first category (0) showed no visible changes. The second (I) was characterized by multifocal spongiosis in the hair follicle epithelium and in the basal epidermis and with mild subepidermal cleft formations. The third (II) category had diffuse spongiosis of the epidermis and with intra- and subepidermal vesicles including eosinophilic material. The fourth (III) showed epidermal and dermal necrosis with destruction of adnexal structures. The relation between morphological changes and treatment doses is presented in Table 6.

Epidermis was significantly thinner after irradiation in the irradiated loin (450 J/cm²) compared to the non-irradiated loin (21.8±8.4 and 30.9±4.2 μm, respectively). Non-irradiated skin (controls) showed a significant variation in thickness of epidermis between fetlock (64.2±13.8 μm), loin (30.9± 4.2 μm) and hamstring (25.3± 2.5 μm) areas (presented as mean and standard deviation).
Table 6. Number of animals with morphological changes after irradiation

<table>
<thead>
<tr>
<th></th>
<th>Hamstring (n=4)</th>
<th>Fetlock joint (n=7)</th>
<th>Loin (n=7)</th>
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</tr>
<tr>
<td>Control</td>
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<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Dose (J/cm²)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
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<tr>
<td>I</td>
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<td>5</td>
<td>3</td>
</tr>
<tr>
<td>II</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>III</td>
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</table>

Categories of morphological changes; 0 = no changes; I = multifocal, mild spongiosis in the basal epidermis with focal subepidermal clef formations; II = diffuse spongiosis in the hair follicle epithelium and of the epidermis with intra- and subepidermal vesicles including eosinophilic material; III = epidermal necrosis with a thin epidermal layer, and coagulation necrosis of underlying dermis.

Clinical evaluation of traumatic arthritis of the fetlock joint (Study IV)

There was no significant difference in the degree of lameness between the laser treated (91 J/cm²) and sham treated group, evaluated by either conventional lameness examination (Table 7) or accelerometer technique. However, there was a weak correlation ($r^2=0.04$) in the degree of initial lameness between conventional lameness evaluation and accelerometer technique. Though not used to evaluate the effect of laser therapy the flexion test was used for diagnostic purposes. The flexion test was judged both subjectively and measured objectively. Here the correlation was considerably better, $r^2=0.66$, as demonstrated in Figure 9 and Table 8.

![Fig. 9. Correlation between flexion test lameness grading assessed by conventional lameness examination (x-axis) and accelerometer technique (y-axis)](image)
Table 7. Comparison of active laser and sham laser groups in respect to clinical outcomes at baseline, after therapy at weeks 1 and 3 (Laser group: 91 J/cm², sham group: 0 J/cm²)

<table>
<thead>
<tr>
<th>Breed</th>
<th>Baseline Lameness score</th>
<th>Week 1 Lameness score</th>
<th>Lameness grading</th>
<th>Complement treatment</th>
<th>Week 3 Lameness score</th>
<th>Lameness grading</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>initial</td>
<td>flexion</td>
<td>initial</td>
<td>flexion</td>
<td>conv</td>
<td>accel</td>
</tr>
<tr>
<td><strong>Active laser group</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Icelandic</td>
<td>0.5</td>
<td>1.5</td>
<td>0.5</td>
<td>1.0</td>
<td>n</td>
<td>0/-</td>
</tr>
<tr>
<td>W.b.</td>
<td>0.5</td>
<td>1.5</td>
<td>0.5</td>
<td>1.5</td>
<td>n</td>
<td>0/-</td>
</tr>
<tr>
<td>Icelandic</td>
<td>0.5</td>
<td>3.0</td>
<td>0.0</td>
<td>0.5</td>
<td>f</td>
<td>+</td>
</tr>
<tr>
<td>Connemara</td>
<td>0.5</td>
<td>2.5</td>
<td>0.5</td>
<td>2.0</td>
<td>n</td>
<td>0/-</td>
</tr>
<tr>
<td>Sib Tr.</td>
<td>0.5</td>
<td>2.5</td>
<td>0.0</td>
<td>0.5</td>
<td>f</td>
<td>0/-</td>
</tr>
<tr>
<td>Pony cross</td>
<td>0.5</td>
<td>3.0</td>
<td>0.0</td>
<td>0.5</td>
<td>f</td>
<td>n.c.</td>
</tr>
<tr>
<td>Russ</td>
<td>0.5</td>
<td>3.5</td>
<td>0.0</td>
<td>1.5</td>
<td>P</td>
<td>+</td>
</tr>
<tr>
<td>W.b.</td>
<td>0.5</td>
<td>2.0</td>
<td>0.5</td>
<td>0.5</td>
<td>n</td>
<td>+</td>
</tr>
<tr>
<td>Median</td>
<td>0.5 (0.5)</td>
<td>2.5 (1.5-3.5)</td>
<td>0.25 (0-0.5)</td>
<td>0.75 (0.5-2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Sham laser group</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sib Tr.</td>
<td>0.5</td>
<td>2.0</td>
<td>0.0</td>
<td>0.5</td>
<td>f</td>
<td>+</td>
</tr>
<tr>
<td>Pony cross</td>
<td>0.5</td>
<td>2.5</td>
<td>0.0</td>
<td>1.0</td>
<td>p</td>
<td>+</td>
</tr>
<tr>
<td>Pony cross</td>
<td>0.5</td>
<td>3.0</td>
<td>0.5</td>
<td>2.0</td>
<td>n</td>
<td>+</td>
</tr>
<tr>
<td>Pony cross</td>
<td>0.5</td>
<td>3.0</td>
<td>0.5</td>
<td>2.5</td>
<td>n</td>
<td>+</td>
</tr>
<tr>
<td>W.b.</td>
<td>0.5</td>
<td>2.5</td>
<td>0.0</td>
<td>0.5</td>
<td>f</td>
<td>+</td>
</tr>
<tr>
<td>Sib Tr.</td>
<td>0.5</td>
<td>1.0</td>
<td>0.5</td>
<td>0.5</td>
<td>n</td>
<td>0/-</td>
</tr>
<tr>
<td>Lipizzaner</td>
<td>1.5</td>
<td>2.5</td>
<td>0.5</td>
<td>2.0</td>
<td>p</td>
<td>+</td>
</tr>
<tr>
<td>Icelandic</td>
<td>0.5</td>
<td>2.0</td>
<td>0.0</td>
<td>0.0</td>
<td>f</td>
<td>+</td>
</tr>
<tr>
<td>Median</td>
<td>0.5 (0.5-1.5)</td>
<td>2.5 (1-3)</td>
<td>0.25 (0-0.5)</td>
<td>0.75 (0-2.5)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Stb Tr. = standardbred trotter; W.b. = warmblooded riding horse; Lameness score 0-5 (0 = no lameness, 5 = non weight-bearing); Lameness grading f = fully improved; p = partially improved; n = not improved; w = worse compared to the beginning of the study; 0/- = not improved; + = improved; conv = conventional lameness examination; accel = accelerometer technique; hyal/cort = hyalurone acid and corticosteroids;¹ = fully improved; ² = not improved at 21-27 days after injection; e = excluded from the study; n.c. = not classified.
As summarised in Table 9, together with own unpublished results from the healthy horses from Study I and II, there were no significant differences found in white blood cell counts and total protein in either blood or synovia. There was no significant difference in the concentration of SP, PGE$_2$ and MEAP in synovia between the laser group and the sham group.

Table 8. Comparison of the flexion test lameness grading by conventional lameness examination and accelerometer technique at initial examination, week 1 and 3 (Laser group 91 J/cm$^2$ and sham group 0 J/cm$^2$)

<table>
<thead>
<tr>
<th>Lameness grading according to flexion test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td>Initial examination</td>
</tr>
<tr>
<td>conv</td>
</tr>
<tr>
<td>------</td>
</tr>
<tr>
<td>Laser group</td>
</tr>
<tr>
<td>Icelandic</td>
</tr>
<tr>
<td>W.b.</td>
</tr>
<tr>
<td>Icelandic</td>
</tr>
<tr>
<td>Connemara</td>
</tr>
<tr>
<td>Stb Tr.</td>
</tr>
<tr>
<td>Pony cross</td>
</tr>
<tr>
<td>Russ</td>
</tr>
<tr>
<td>W.b.</td>
</tr>
<tr>
<td>Sham group</td>
</tr>
<tr>
<td>Stb Tr.</td>
</tr>
<tr>
<td>Pony cross</td>
</tr>
<tr>
<td>Pony cross</td>
</tr>
<tr>
<td>Pony cross</td>
</tr>
<tr>
<td>W.b.</td>
</tr>
<tr>
<td>Stb Tr.</td>
</tr>
<tr>
<td>Lippizaner</td>
</tr>
<tr>
<td>Icelandic</td>
</tr>
</tbody>
</table>

Stb Tr. = standardbred trotter; W.b. = warmblooded riding horse; Lameness score 0-5 (0 = no lameness, 5 = non weight-bearing); Accelerometer grading 0/- = not improved compared to initial examination; + = improved compared to initial examination; conv = conventional lameness examination, accel = accelerometer technique; e = excluded; n.c. = not classified.
Table 9. Results from blood and synovia analyses in sound horses and horses with traumatic arthritis of the fetlock joint irradiated with active laser or sham laser (Laser group = 91 J/cm², sham group = 0 J/cm²)

<table>
<thead>
<tr>
<th></th>
<th>Sound horses, one irradiation</th>
<th>Injured horses, five irradiations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Laser (n=10) (n=12) Before</td>
<td>After</td>
</tr>
<tr>
<td><strong>Blood</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B-LPK 10x9/L</td>
<td>7 (4-10)</td>
<td>7 (5-10)</td>
</tr>
<tr>
<td>S-Protein g/L</td>
<td>61 (2-73)</td>
<td>62 (3-71)</td>
</tr>
<tr>
<td><strong>Synovia</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leuk 10x6/L</td>
<td>156 (46-605)</td>
<td>275 (4-3310)</td>
</tr>
<tr>
<td>Protein g/L</td>
<td>8 (5-11)</td>
<td>18 (5-36)</td>
</tr>
<tr>
<td>Substance P fmo/l/ml</td>
<td>28 (12-69)</td>
<td>32 (5-87)</td>
</tr>
<tr>
<td>MEAP fmo/l/ml</td>
<td>104 (75-203)</td>
<td>122 (94-191)</td>
</tr>
<tr>
<td>PGE2 pg/ml</td>
<td>746 (15-9935)</td>
<td>576 (15-3336)</td>
</tr>
</tbody>
</table>

B-LPK = Blood leukocytes; S-Protein = Serum protein; Leuk = Synovia leukocytes; Protein = Protein in synovia; MEAP = Met-enkephalin-Arg-Phe; PGE2 = prostaglandin E2.

Data is presented as medians and range. Two groups of sound horses: standing (91 J/cm²) and anaesthetised (91-137 J/cm²).
Discussion

The general aim of the present investigation was to evaluate the thermal effects of defocused CO₂ laser on equine tissues by use of objective methods also applicable to other rehabilitation modalities. The reasons for focusing on the thermal effects were twofold. Firstly, the kinetic excitation of the defocused CO₂ laser results in a photothermal effect (Hecht, 1992; Andersson, 1994). Secondly, many of the postulated effects of high-power laser therapy, such as increased blood perfusion and modulation of pain, have an increase in temperature as the proposed mode of action. The clinical effects were studied on horses with traumatic arthritis of the fetlock joint, a diagnosis chosen because of its pathophysiological manifestation with pain and inflammation, for which laser therapy is postulated by some authors to have a positive effect (Baxter, 1994; Campana et al., 2004; Bjordal et al., 2006). Besides being a suitable injury to treat, the fetlock arthritis was also registered as the most common condition in Swedish riding and leisure horses (Penell et al., 2005), consequently the choice of condition also had both an animal welfare and an economic aspect.

Physiological effects of defocused CO₂ laser irradiation on temperature, blood flow and skin morphology

The application of therapeutic heat is often part of a rehabilitation program. The therapeutic goal is to achieve a sufficient rise in temperature at the site of injury, without causing any negative side effects such as thermal injuries. The heat can be administered superficially or to deeper situated tissues as in therapeutic ultrasound. Due to its absorption pattern, the defocused CO₂ laser can be classified as superficial heat.

It is generally accepted that heat modulates pain perception and stimulates regeneration of injured tissue. A proposed mechanism of action is an increased tissue temperature with secondary increase in blood flow. This increase in blood flow (Löfgren et al., 1997) may help removing inflammatory agents from the injured location, thus reducing the sensitisation of mechanoreceptors. Furthermore, a high tissue temperature directly influences nerve transmission (Klumpp & Zimmermann, 1980; Lehmann, 1990; Wesselmann et al., 1991) and the sensory stimulation may influence the pain modulation by activation of pain inhibitory systems (Melzack & Wall, 1965; Sluka et al., 1999; le Bars, 2002).

In the present study, the temperature increase of at least 3-6 °C in skin and subcutis could be classified as a moderate to vigorous heating effect according to Lehmann (1990).

No significant change in the temperature was observed in the fetlock joint. Previous studies have shown both an increase (Weinberger et al., 1988; Osterweld et al., 1994a; Osterweld et al., 1994b) and a decrease (Hollander & Horwath, 1949) in joint temperature in response to therapeutic heat. Use of heat in acute injuries is not recommended, as a higher temperature may result in an increase in blood flow, causing an oedema in soft tissues (Lehmann, 1990) and a possible activation of cartilage-degenerative enzymes (Castor & Yaron, 1976).
Weinberger et al., 1989). The unaffected joint temperature in the present study indicates a possibility to use defocused CO$_2$ laser irradiation without increasing the risk to activate cartilage-degenerative enzymes.

We found a rise in skin blood flow, but not in the temperature or blood flow in muscle. This could be due to either the limited penetration depth of the laser light (Carruth & McKenzie, 1986) and/or a sufficient temperature regulation due to vascular heat dissipation in the skin. This is in accordance with other studies on superficial heat where both the temperature and blood flow in muscle remains relatively unaffected (Johnson et al., 1976).

Studies on laser therapy with “near” infrared light has reported a non-photothermally mediated vasodilatation in the skin of rats (830 nm, 185 J/cm$^2$, Kubota, 2002) and humans (780 nm, 5 J/cm$^2$, Schaffer et al., 2000). In the present study, the increase in skin blood flow was observed shortly after the onset of irradiation and immediately after the first rise in temperature, thus suggesting that the increase in flow could be caused by neuronal reflexes. It is likely that an axon reflex was activated since the increase in blood flow appeared fast and was detected almost immediately at a distance of 3 cm from the irradiated. There are studies showing that the axon reflexes can be activated by non-painful thermal stimulation (Minson et al., 2001; Stephens et al., 2001) in the interval of 30-42 °C (Barcroft & Edholm, 1943; Taylor et al., 1984; Johnson et al., 1986; Magerl & Treede, 1996). One can assume that both warmth receptors and heat-sensitive nociceptors, with a threshold of approximately 40 °C (Torebjörk et al., 1984; Tillman et al., 1995), were activated during our investigation. The activation of heat-sensitive nociceptors has two important consequences. It can contribute to the inflammatory response through the release of inflammatory substances and with a secondary release of opioids (Sprengler et al., 2006) it may down-regulate the inflammatory response. A painful stimulus activates the withdrawal reflex, protecting the tissue from thermal injuries. It is therefore important that the horse can perceive the pain signal (i.e. that it is not heavy sedated) to be able to react on too high doses. In our studies, we observed a high increase in skin temperature in unclipped long hair coat compared to clipped skin after irradiation with 171 J/cm$^2$, indicating a higher risk for thermal injuries when irradiating horses with long hair coats. This is in accordance with a study on surface temperature in dogs receiving therapeutic ultrasound (Steiss & Adams, 1999). However, there was no significant difference in temperature increase between horses with short hair coat and clipped coat, receiving a lower irradiation dose (91 J/cm$^2$). This might be due to the lack of insulating properties of the short or clipped hair coat.

The temperatures recorded in our study were measured at 1 and 3 cm from the irradiated area. It is most likely that the temperature at the irradiated site was higher, thus activating more heat-sensitive nociceptors. This assumption is based on studies using pulsed surgical laser irradiation (Fried et al., 1999; Osmond et al., 2000) in which the temperature was measured directly at the irradiated area and stepwise at distances of 6 and 7 mm from that area. A difference up to 30 °C was noted between the irradiated area and about 1 cm from there. The assumption is also supported by the morphological changes observed in the present study. The threshold for tissue protein denaturation is a function of temperature and heating time (Moritz & Henriques, 1947), and some of the observed morphological changes indicate tissue temperatures well above 45 °C. It is most likely that the
morphological changes are different degrees of thermal injury, since similar changes are reported from other studies (Laor et al., 1969; Zweig et al., 1990).

Thermal injury may also cause an inflammatory reaction, which activates a complicated cascade of reactions in the immune and vascular systems, inducing a vasodilatation (Löfgren et al., 1997). The purpose of the inflammatory reaction is to initiate healing of damaged tissue. It has been suggested that the tissue injury acts as a counter-irritant, which activates the central diffuse noxious inhibitory system and thereby reduces pain (Le Bars, 2002). The laser beam in the present studies was scanned over the irradiation area, which creates a quasi-continuous delivery of radiation with pulse duration of approximately 0.07 s. With a pulse duration longer than the tissue temperature relaxation time, there is a risk for thermal injury due to heat conduction (Walsh et al., 1988; Zweig et al., 1990). Our results showed dose-dependent changes in skin morphology after defocused CO₂ laser irradiation with all three different doses, ranging from therapeutic to near-surgical.

Some changes were observed below estimated laser absorption depth, which indicates heat conduction in the tissue. This was especially relevant for the differences in morphological changes observed after the 91 and 137 J/cm² doses (with more changes and at a greater depth after the 137 J/cm² dose), since the only factor that distinguished the doses from each other was the irradiation time, i.e. the number of scanning repetitions. Notably, the morphologic changes after the 137 J/cm² dose were not correlated to any macroscopic changes observed within 90 min after irradiation.

The doses that caused the moderate to severe changes (137 and 450 J/cm² doses) are, to our knowledge, not normally used in bio-modulating CO₂ laser therapy, but can be attained by accidentally reducing the irradiation area.

In the present study the temperature in the centre of the irradiation area was not measured. It would have been desirable to have temperature probes designed for direct measurement. We can assume that the temperature at the irradiation area was at least higher than those recorded at 1 cm from the irradiated area. It would also have been desirable to measure changes in blood flow on standing horses, but experience shows that the movement artefacts would have limited the possibilities to draw any conclusions from the recordings (Edner, 2005).

The present physiological effects caused by defocused CO₂ laser irradiation may be compared to other therapeutic heat modalities: warm water (42-45 °C) from a water hose increased the skin temperature by approximately 10 °C (Kaneps, 2002). A gel wrap heated to 40 °C and applied to the metacarpal region for 30 min raised skin temperature by 5 °C, and therapeutic ultrasound has been shown to cause a difference of less than 1 °C (1.5 W/cm²) between treated and untreated limbs in horses (Turner & Wolfsdorf, 1991).

Due to the limitations of the LDF technique caused by different experimental setups, it is difficult to compare the effect on blood flow with other reports. However, studies on transcutaneous electric nerve stimulation and acupuncture in humans show a two to threefold increase in the microcirculation in the skin (Cramp et al., 2002; Kuo, 2004) and tenfold in muscles during muscle activity (Sjaastad et al., 2003).
Effects of defocused CO$_2$ laser irradiation on traumatic arthritis of the fetlock joint

The clinical study did not reveal any significant difference in the reduction of lameness between horses treated with defocused CO$_2$ laser (91 J/cm$^2$) for traumatic arthritis of the fetlock joint compared to a sham group horses. Nor was there a significant difference between the groups in the results from the blood and synovia analyses. However, considering the physiological effects of temperature, blood flow and morphology observed in our experimental studies it is important to consider possibilities why the treatment did not result in a difference in detectable clinical improvement.

Laser studies on osteoarthritis in humans have shown relief of pain (Nivbrant & Friberg, 1992; Bertolucci & Grey, 1995; Basford et al., 1999; Gur et al., 2003; Bjordal et al., 2003), or no differences between treated and controls (Basford, 2000; Brosseau et al., 2004; Tascioglu et al., 2004; Brosseau et al., 2005). The lack of confirmed mechanisms of action and the diversity of clinical outcome generate uncertainties about proper dosage and treatment indications. When planning the present study, no recommended standard therapy programs regarding dose and duration of the laser treatment were available for horses. This is also the case with laser therapy in humans, where no consensus has been reached on optimal treatment parameters (Basford, 1995; Tuner & Hode, 1998; Basford et al., 2000; Bjordal et al., 2003; Nussbaum et al., 2003) or conclusive evidence of dose-dependency has been shown (Bjordal et al., 2003; Gur et al., 2003; Naeser, 2006). The dose used in the present study mimics clinical practice and was promoted by the manufacturer of the laser system and calculated in order to achieve a possible therapeutic dose in the fetlock joint. Based on the results from the studies on temperature, blood flow and skin morphology, a decrease in dose would likely limit the physiological effects leading to a reduced clinical effect, while an increase in dose could hazard the condition of the superficial tissues. Only one joint in each horse was treated, although some of the horses had bilateral traumatic arthritis of the fetlock joint. It has been proposed that laser treatment unilaterally may induce similar changes in the contra-lateral site (Rochkind et al., 1989). It is unlikely that there was a major systemic effect of the laser irradiation, since there was no difference in the blood parameters analysed.

Relatively few horses met the inclusion criteria, but the study was randomised, blinded and controlled. The horses were examined by the same experienced clinician using the same protocol. Based on preliminary power analysis calculations, our aim was to include 30 horses in the study. In an early stage of the trial, after experience difficulties in recruiting suitable horses, a discussion on changing inclusion criteria was held, and it was decided to widen the criteria to include bilateral forelimb lameness. Other changes in the protocol, such as exclusion of the control group or allowing other lameness’s, were rejected since that would have negatively influenced the scientific quality. The study was then re-started. In total, 45 horses were examined and due to strict inclusion criteria, 16 horses were accepted. The partly inconclusive results may be caused by the sample size.

In our material, the improvement rates in both groups were approximately 50%.
These results may be compared to an earlier prospective study on defocused CO₂ laser therapy on traumatic fetlock arthritis in horses (Lindholm et al., 2002) which reports an improvement rate of 80% after laser treatment, compared to 68% after intra-articular injection of bethametasone together with sodium hyaluronate. In the observer-blind part of the referred study, improvement was confirmed in 3/5 horses (60%) in both groups after 3-5 weeks. To our knowledge, no studies have been published on the clinical effects of other types of therapeutic heat in horses, and there is a lack of scientific studies on different rehabilitation modalities. However, studies on other conventional treatments of traumatic or induced arthritis show an improvement or return to health at approximately 3 weeks that differs between 45% (10/22 horses) receiving intra-articular injection of 0.9% NaCl (Gaustad et al., 1999), 52% (21/41) of horses treated with sodium hyaluronate (Verschooten & Desmet, 1997), 69% (38/55) for polysulphated glucosaminoglycan or sodium hyaluronate (Gaustad & Larsen, 1995), 12 (1/8) to 80% (49/61) for corticosteroids (Ryдел et al., 1970; Verminib et al., 1977) and “excellent” for 83% (10/12) of the horses receiving the combination sodium hyaluronate and corticosteroids (Ryдел et al., 1970). In a study by Gaustad et al. (1999) the improvement rate for rest alone was 56% (9/16 horses). In the present study, it is possible that an effect from the laser therapy have not been able to be separated from a self-healing effect. It would be interesting to extend the follow-up period in order to see if there are any delayed effects not detected 3 weeks after the initial examination.

The previously mentioned studies comprised different assessment tools and the horses exhibited different degrees of lameness. Since traumatic arthritis has both a pain and an inflammatory aspect, and assessment of pain is often done by examining the degree of lameness, the evaluation in the present study was complemented by an objective accelerometer technique, as well as analyses of SP, PGE₂ and MEAP in synovia. The lameness evaluation was based on comparison of changes in initial lameness. Contrary to some of the previously mentioned studies, the flexion test was used only as a diagnostic tool and not primarily in the evaluation of the degree of lameness. This choice was based on studies showing that the response of a forelimb flexion test may vary with time, with the clinician performing the test and from day to day, and may not be well correlated with radiological findings or future occurrence of forelimb lameness (Ramey, 1997; Verchooten & Verbeeck, 1997; Busschers & van Weeren, 2001). However, if the evaluation had instead been done on the outcome of the flexion test (improvement defined as a reduction in the degree of lameness) all horses but two would have been classified as improved, however without any significant difference between laser and sham treatment.

An accelerometer technique is a method earlier described and proven as an objective method for lameness evaluation (Weishaupt et al., 2001; Keegan et al., 2002; Lelé et al., 2004). However, in the present study the correlation between the grading of the clinician and the accelerometer data was weak. These results are contrary to previous studies in which a symmetrical vertical head movement was lost at forelimb lameness (Barrey & Desbrosse, 1996; Vortenbosch et al., 1997) and the degree of asymmetry detected by accelerometer technique was related to the degree of lameness confirmed by conventional lameness examination.
Weishaupt et al., 2001) The reason may be the low variation in the conventionally assessed degree of initial lameness in the horses entering the study. On the other hand, there was good correlation between the grading of the clinician and the accelerometer data of the flexion test when the variation in the grading was higher. Our conclusion is that the accelerometer technique was a valuable complement to get a more complete evaluation of low-grade initial lameness and that it enhance the validity of the conclusions.

It is reasonable to assume that pain in traumatic arthritis may have synovitis as a major origin (Howard & McIlwraith, 1996). As we have measured effects on superficial tissue temperature and blood flow indicating a sensory stimulation, which possibly has a pain reducing, and an anti-inflammatory effect, analyses of the inflammatory mediator SP, PGE2 and the opioid MEAP in synovia were performed. The opioid MEAP is a neuromodulator, important in anti-nociception and inflammation (Kiser et al., 1983; Rosen et al., 2000) and a recently published study reports on an opioidergic activation in the medial pain system after heat stimulation (Sprengler et al., 2006). Contradictory results have been reported in studies on human cerebrospinal fluid, with a lower concentration of Met-enkcephalin in patients with chronic pain compared to those without pain (Simmonet et al., 1986) and higher levels of MEAP in patients with fibromyalgia (Baraniuk et al., 2004). The concentration of met-enkephalin has been detected in pituitary effluent blood, peripheral blood and cerebrospinal fluid in horses (Luna et al., 1998). Another study on horses report on opioid receptors in the synovial membranes and that opioids can decrease inflammatory-induced pain through inhibition of the release of SP from peptidergic neurons (Sheehy et al., 2001). However to our knowledge, no studies have been published on MEAP data in horses. Our unpublished results show a significant difference in the concentration of MEAP between sound horses (one group of standing and one group of anaesthetised horses) and horses with traumatic arthritis of the fetlock joint, with a significantly lower concentration in the synovia of the lame horses (p<0.001).

Although, the intra-assay variance was relatively high, it is still likely that our results are accurate as all samples were randomly ordered, assayed the same day and tested in the same RIA. However not significant (p<0.14), the concentration of MEAP was increased in 5 out of 7 horses in the laser group compared to 2 out of 5 horses in the sham group. In the present study, the tendency towards increased MEAP may suggest a possible pain modulating effect, correlated to the clinical status of the horses in the laser group.

An anti-inflammatory effect has earlier been shown after laser irradiation (Amano et al., 1994; Ulugöl et al., 1997; Campana et al., 1999; Bjordal et al., 2006). No significant difference in the concentration of SP or PGE2 was observed between groups, or between sound horses and horses with traumatic arthritis. However, other studies have shown a higher concentration of the pro-inflammatory SP and PGE2 in synovia from horses with joint disease compared to normal joints (Caron et al., 1992; May et al., 1994; Gibson et al., 1996; Owens et al., 1996; Hawkins et al., 1993; Hardy et al., 1997; Kirker-Head et al., 1999; Bertone et al., 2001). It is likely that relatively low concentrations measured in the lame horses reflect the severity of the traumatic arthritis since the horses exhibited a low grade lameness.
Summary and conclusions

Objective outcome measurements such as Laser Doppler Flowmetry and temperature recordings together with accelerometer technique to examine lameness proved to be valuable when evaluating a rehabilitation modality such as the defocused CO₂ laser. It is likely that these methods can be of equal value when assessing the effect of other physical rehabilitation modalities used in veterinary medicine. The hypothesis that defocused CO₂ laser irradiation reduces the degree of lameness, due to traumatic arthritis of the fetlock joint, could not be verified.

Irradiation with defocused CO₂ laser resulted in changes of the following parameters:

Local temperature
- an increased temperature in skin and subcutis
- an accumulation of heat in the subcutis
- a greater increase in temperature in unclipped compared to clipped skin
- no changes in the temperature of the fetlock joint or in muscle

Local blood flow
- an increased blood flow in skin
- no changes in the blood flow in muscle

Skin morphology
- no to mild morphological changes for the 91 J/cm² dose
- mild to moderate morphological changes for the 137 J/cm² dose
- moderate to severe morphological changes for the 450 J/cm² dose

Blood and synovia
- a higher concentration of MEAP in synovia in healthy horses compared to horses with traumatic arthritis of the fetlock joint
- no difference in the concentration of SP, PGE₂ and MEAP in synovia between laser and sham treated lame horses

Clinical effects
- no difference in the degree of lameness between the active laser treated (91 J/cm²) and sham group horses, evaluated by either conventional lameness examination or accelerometer technique
Clinical implications

The present investigation was undertaken to study physiological effects of defocused CO_2_ laser irradiation and assess the therapeutic value in horses with traumatic arthritis of the fetlock joint.

The results showed that irradiation with defocused CO_2_ laser with our specified doses had a photothermal effect, causing an increase in temperature in superficial tissues. The increase in skin temperature was greater in unclipped horses compared to clipped, indicating a greater risk for thermal injuries when irradiating horses with a long hair coat. The increase in skin and subcutis was of such intensity that restrictions are suggested on the use of irradiation on dermal injuries in the acute stage, in order to avoid the risk for oedema. There was no increase in the temperature in the fetlock joint, which indicates that it may be possible to irradiate traumatic arthritis without activating cartilage-degenerative enzymes.

The photothermal effect also involves a risk of thermal injuries. The blood flow in muscle may be stimulated by a higher dose than 91 J/cm^2, but the risk of thermal injuries in skin is obvious. Whenever possible, more efficient methods, such as active muscle work, is preferable to increase blood flow in muscles.

The risk of negative side effects increases with an accumulation of heat when the laser is applied with insufficient relaxation time between irradiations or between each scanning repetition. A power density that normally does not induce morphological changes may do so when repeated. Consequently, there were more morphological changes in the skin after irradiation with 137 J/cm^2 (16 W, 6 x 7 cm, 6 min) compared to 91 J/cm^2 (16 W, 6 x 7 cm, 4 min). Therefore, the use of an average dose higher than 91 J/cm^2 on equine skin should be considered carefully. It is important to be aware of the risk of significant microscopic damage without simultaneous macroscopic changes after laser irradiation. Since the increase in temperature is correlated to the degree of thermal injury, it is essential that the treatment set-up permits the horse to withdraw the irradiated limb if the treatment becomes painful. Therefore heavy sedation of the horse before treatment should be avoided.

Finally, it is essential to handle the CO_2_ laser device correctly. The effect of the laser should be monitored regularly by use of an external output power detector as the mirrors reflecting the laser beam easily becomes dusty, and consequently give a lower dose than expected. Measurement of the irradiation area on the target should also be closely monitored, as an accidentally reduced area of treatment increases the irradiation dose and endangers the welfare of the horse.
**Future research**

Further research should focus on validating rehabilitation assessment tools. Most important, further studies on pain assessment are necessary in order to evaluate rehabilitation modalities, as a majority of the modalities in one way or another focuses on pain relief.

One main outcome effect of a successful treatment on musculoskeletal injuries is the reduction of pain, evaluated as a reduction in lameness. It is therefore important to refine and develop the accelerometer technique, in order to objectively detect and confirm subtle changes in movement at both training and clinical settings.

Many of the modalities used in rehabilitation have an explanatory model based on increased blood flow. Thus it would be of great interest to refine a non-invasive Laser Doppler Flowmetry technique for intramuscular blood flow measurements on standing animals.

Further research on physical rehabilitation modalities should focus on defining modes of action and validating treatment protocols. Before applying defocused CO₂ laser treatment in clinical practices, it is important to continue studies on morphology with alternative doses and treatment locations, as well as extending the present clinical study on more horses and to evaluate clinical effects on other types of injuries.
References


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Populärvetenskaplig sammanfattning

Intresset för rehabilitering har ökat markant inom veterinärmedicinen under de senaste åren. Målet med rehabilitering är att återställa bästa möjliga funktion efter skada eller sjukdom. Vid rehabiliteringen används olika fysikaliska behandlingsmetoder, däribland behandling med defokuserad CO₂ laser. Vid sådan behandling omvandlas laserjusets energi till värme i vävnaden. En ökad temperatur anses kunna lindra smärta samt påskynda läkning av olika vävnader, och värmeehandling har därför använts sedan urminnes tider.

I denna avhandling har de fysiologiska effekterna av behandling med defokuserad CO₂ laser undersökt i fyra olika studier: tre experimentella och en klinisk. Syftet har varit att studera om behandlingen resulterar i en värmeökning, och – om så är fallet – om ökningen åtföljs av ett förhöjt blodflöde, om en ökning av värme och blodflöde leder till smärtlindring och steggrad läkningstendens samt om värmeökning kan påverka hudens mikroskopiska bild.

Temperatur, blodflöde och morfologiska förändringar i huden har studerats på friska vakna och sövda hästar. Resultaten visar att laserljuset gav upphov till en värmeökning i huden och underhuden, medan temperaturen i muskler och kotleder föreblev opåverkad. Temperaturökningen i huden orsakade beroende på dos laserljus (energi/kroppsyta) milda till kraftiga mikroskopiska förändringar, som liknar dem man kan se vid bränskedamor. Temperaturökningen kvarstod längre i underhuden än i huden och den blev också större hos hästar med lång härrem än hos dem med klippt päls. Laserbehandling påkunde blodflödet i huden, men inte i underliggande muskulatur.

I den kliniska undersökningen studerades laserbehandlingens smärtlindrande och antiinflammatoriska effekter genom att hästar med traumatisk ledinflammation i kotleden undersöks. Som kontrollgrupp användes också hästar som fått placebobehandling. Smärtlindring mätts i detta sammanhang ofta genom att man undersöker om det skett en minskning i graden av hålta, eftersom hästens subjektiva smärtupplevelse inte kan fastställas. I studien användes både konventionell håltbedömning utförd av en erfaren kliniker och objektiv mätning av rörelsen med ett accelerometersystem. Dessutom mättes förändringar i koncentrationen av markörer för smärta och inflammation i ledvätska före och efter behandling. Resultaten visar att hästarna blev bättre efter behandlingsperioden, men att det inte var någon skillnad mellan de hästar som fått laserbehandling och de som fått placebo. Det var heller ingen skillnad i koncentrationen av markörer mellan grupperna.

Effect of defocused CO₂ laser on equine skin, subcutis and fetlock joint temperature

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Abstract

Despite the increasing use of lasers in the rehabilitation of horses, the biophysical action of the laser is not clearly defined. The purpose of this study was to determine the effect of a defocused CO₂ laser on the temperature of the skin, subcutis and fetlock joint in standing and anaesthetized horses. A cross-over design comprising 10 standing horses was used. Consecutive irradiation (91 J cm⁻²) was applied to each of the three aspects of the front fetlock joint of these animals. In 12 anaesthetized horses (eight laser-treated and four control), irradiation (137 J cm⁻¹) was applied to the dorsal side of the joint. In the standing group, skin temperature increased on average by 5.3 ± 8°C to 34.8 ± 1.5°C (P, 0.05) and the subcutis temperature increased by 5.7 ± 8°C to a mean temperature of 36.0 ± 0.9°C during laser treatment. There was no difference in joint temperature between laser-treated and control horses. Similar results were obtained in anaesthetized horses. Treatment with a defocused CO₂ laser caused a significant increase in the temperature of the skin and subcutis, but not in the joint cavity. Further studies are needed to investigate if the increase in temperature influences perfusion and modulation of pain, as a result of defocused CO₂ laser treatment.

Keywords: defocused CO₂ laser; horses; laser therapy; thermal effect; rehabilitation

Introduction

The use of physical therapy is a rapidly growing field in horse rehabilitation. One modality that has gained interest is the laser. It is used, in both humans and animals, in the treatment of a variety of injuries such as wounds, tendonitis, back problems and osteoarticular diseases. Relatively few studies describe the outcome of these treatments. However, results from a recent study indicate that a defocused carbon dioxide (CO₂) laser may be an applicable treatment for acute synovitis in horses.

The effect of laser radiation on tissue structures and function remains unclear. The CO₂ laser is proposed to produce photochemical and photomechanical, as well as thermal effects. These photothermal effects result from the transformation of absorbed light energy to heat. It has been suggested that thermal effects initiate pain relief, increase tissue perfusion and reduce muscle spasms, explanations shared with other thermal modalities used in physical rehabilitation.

The goal of physical therapy is to promote the healing of tissues through the stimulation of normal physical processes, thereby restoring the function of injured tissues. Physical therapy makes a distinction between ranges of temperatures considered to have a beneficial influence on human tissue. Therapeutic effects are expected in the approximate temperature range 40 to 45°C. In addition, the therapeutic effects are also dependent on the increase in heat and the area heated. Based on earlier studies on human muscles, an increase of 1°C (mild heating) accelerates the

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metabolic rate in tissues. An increase of 2–4°C (moderate heating) reduces muscle spasm, pain and chronic inflammation, and increases blood flow, while vigorous heat (≥ 4°C) decreases the viscoelastic properties of collagen and inhibits sympathetic activity.

Extreme temperatures above 45°C may cause thermal pain and injury to tissues. Repeated exposures, individually incapable of causing recognizable epidermal injury, may have the same total destructive capacity as a single exposure of longer duration.

Intra-articular temperature is suggested to increase by deep-heat treatments such as therapeutic ultrasound and decrease by superficially applied hot packs. Contrary to this, results from studies on humans with arthritis indicate that the intra-articular temperature is increased by superficial heat and the general consensus is that superficial heat may aggravate symptoms in acute inflammatory arthritis.

The activity of cartilage-degrading enzymes in human rheumatoid arthritis and active osteoarthritis is influenced by local joint temperature. The destruction of articular cartilage by collagenase is shown to be significant at 37°C and very low at 32°C. There-fore, treatments that elevate intra-articular temperature, such as superficial heat and ultrasound treatment, are unsuitable for treating active arthritis or secondary synovitis in osteoarthritis.

When designing a rehabilitation programme with a thermal modality such as the defocused CO2 laser, it is important to predict both benefits and risks of the thermal exposure. The aim of the present study was to investigate the effects of defocused CO2 laser treatment on temperature in the skin, subcutis and fetlock joint in the horse. The hypothesis was that laser treatment would result in an increase in temperature in the skin, subcutis and fetlock joint. Further, a more pronounced temperature increase was expected in skin that had an unclipped hair coat, compared with clipped.

**Materials and methods**

**Laser equipment**

A defocused CO2 laser (KSV 25S, EL.EN. Srl, Florence, Italy) was used in the study. The HeNe source emitted continuously at 1.2 mW. The laser was calibrated regularly, and an external detector (LaserMate Detector, COA-35-0191-000, Gamma Optronic AB, Uppsala, Sweden) was used to measure the output effect before and after each treatment.

**Temperature protocol**

The temperature in the skin, subcutis and fetlock joint was measured using a thermistor with a range between −1 and +50°C (DM852; ELLAB, Rødovre, Denmark). A flexible probe 0.8 mm in diameter (MAA-08 500-A; ELLAB) was inserted 3–4 cm into the fetlock joint, from the lateral aspect. A needle probe measuring 0.8 mm in diameter (MKA-08 050-A; ELLAB) was inserted approximately 15 mm under the skin, in the midline of the dorsal side of the fetlock joint and 2 cm proximal to the treatment area. A skin probe (MHB-08025-A; ELLAB) was attached with adhesive tape over the midline, 2 cm proximal to the treatment area. After each treatment occasion, the thermistor and the probes were controlled in a water bath using a mercury thermometer.

**Horses, experimental environment and experimental design**

The experimental protocol consisted of one study on standing horses and three studies on anaesthetized horses. The experiments were approved by the Ethical Committee on Animal Experiments in Uppsala, Sweden.

**Standing horses**

Ten healthy Standardbred trotters (four females and six geldings) with a mean weight of 489 kg (range 403–580 kg) and a mean age of 7 years (range 3–13 years) were used in the study.

The experiment was designed as a cross-over study with randomized laser and control treatments (with no laser output). On each treatment occasion, body temperature was measured and a blood sample was drawn from the jugular vein. An area of 6 cm × 7 cm on the lateral, dorsal and medial sides of the fetlock joint was washed with antiseptic solutions (Hibitan, Zeneca, Göteborg, Sweden). Approximately 20 min was allowed between the antiseptic wash and the start of the procedure. Consecutive irradiations of 91 J cm$^{-2}$ (16 W) were applied at a distance of 1 m on the lateral, dorsal and medial aspects of the fetlock joint for 4 min, respectively. Temperature was measured at 30-s intervals; from 5 min before the start of the treatment, during the treatment and for 5 min after the end of the treatment.

**Anaesthetized horses**

This first study on anaesthetized horses consisted of 12 healthy Standardbred trotters (11 females and one gelding) with a mean weight of 501 kg (range 375–580 kg) and a mean age of 7 years (range 2–20 years). Eight horses received laser treatment and four served as controls.

Laser-treated and control groups were randomized. Body temperature was measured and a blood sample was drawn from the jugular vein. The horses were anaesthetized according to two different protocols; animals were pre-medicated with either acepromazine (Plegicil vet.; Pharmacia Upjohn Animal Health, Sweden) or one of the following: acepromazine (0.2 mg/kg body weight, i.m.) and detomidine (0.02 mg/kg body weight, i.m.) or detomidine alone (0.01 mg/kg body weight, i.m.).
Anaesthesia was induced intravenously with guaifenesin (Myolaxin vet.; Orion Pharma, Finland). Anaesthesia was induced intravenously with halothane (Fluotane vet.; Chassot & Cie AG, Switzerland) and thiopentone (Pentothal natrium 12.5%; Abbott, Solna, Sweden). The horses were intubated, transported to the surgical table and placed in left lateral recumbency. The non-dependent forelimb, which was used for treatment, was supported in a position perpendicular to the body axis. Anaesthesia was maintained with either halothane (Fluotane8; Astra, Södertälje, Sweden) or isoflurane (Forene®; Abbott) in oxygen. An electrolyte solution (Ringer acetate; Pharmacia & Upjohn: P&U) was continuously infused through a catheter in the left jugular vein. Spontaneous breathing was allowed from a semi-closed, large-animal circle.

The treatment area (6 cm $\times$ 7 cm) on the dorsal side of the fetlock joint area was washed with an antiseptic solution and the temperature probes were introduced as described above after synovia had been sampled. The irradiation energy was 135 J cm$^{-2}$ (16 W, 6 min) and the temperature was measured at the similar intervals as in the standing horse, from 5 min before start of the treatment, during the treatment and until 30 min after the end of treatment (recordings were carried out every minute from 10 min after end of treatment).

**Study on temperature in clipped and unclipped hair coat (clipped/unclipped)**

A second experiment was performed on seven of the anaesthetized horses described earlier, four females and three geldings, with a mean weight of 464 kg (range 375–548 kg) and a mean age of 9 years (range 2–24 years). The experiment was designed as a cross-over study and the two treatments, laser on clipped and unclipped skin, were randomized. A treatment area (6 cm $\times$ 7 cm) over each gluteal muscle was prepared, one part clipped and one with the hair coat intact. A small area for the measuring probes, in direct contact with the treatment area, was also clipped and surgically prepared, and a skin temperature probe and a probe inserted subcutaneously were attached. The irradiation energy was 171 J cm$^{-2}$ (20 W, 6 min) and the temperature was measured at the same intervals as in the standing horse: from 5 min before start of the treatment, during the treatment and until 5 min after the end of treatment.

**Extended study on fetlock temperature during anaesthesia (extended study)**

The third study comprised six, healthy Standardbred trotters (four females and two geldings) with a mean weight of 483 kg (range 460–507 kg) and a mean age of 8 years (range 5–13 years). Four horses received laser treatment and two served as controls. The laser-treated and control groups were randomized. The procedure was similar to the protocol used in the anaesthetized group except that all horses were pre-medicated with detomidin (Domosedan® vet.; Orion Pharma), placed in dorsal recumbency, and that anaesthesia was maintained with isoflurane (Forene®; Abbott) in oxygen. An output power of 16 W for 4 min (irradiation energy of 91 J cm$^{-2}$) on the dorsal side of the fetlock joint was used. The treatment protocol started when the temperature had reached a steady state, and ended when a new steady state was observed after treatment. Steady state was defined as 10 min of stable temperature ($\pm$ 0.1°C).

The temperature of the joint was recorded every minute, during the 4 min of treatment and continued until steady state after treatment.

**Statistical analysis**

For data analysis, Statistica 6.0 (Statsoft, 2001; Statsoft Scandinavia AB, Uppsala, Sweden) was used and results are presented as mean $\pm$ standard error. Statistical significance was accepted at $P < 0.05$. The intention of the study was to describe the total heating effect during a specific time period from a relatively small amount of experimental material, i.e. the cumulative response to the intervention. Therefore, the method ‘area under curve’ was selected.

Before the statistical calculations, the data were individually corrected by subtracting the mean for each individual pre-treatment period. The area under the curve for the total treatment period, as well as for each treatment projection itself (i.e. lateral, dorsal and medial projection), was calculated. Wilcoxon matched pairs signed rank sum and Mann-Whitney tests were used where appropriate.

**Results**

**Standing horses**

The temperature response to laser treatment is shown in Table 1. The results presented for skin and fetlock joint temperatures are from nine horses, since the results from the measurements of one horse had to be excluded due to failure of the temperature probe (Fig. 1).

The temperature response in the skin is illustrated in Fig. 2a. The increase in temperature was significantly higher during laser treatment compared with the controls. The maximum temperature was reached in the dorsal projection during treatment, followed by a decline towards the baseline level. The average increase, measured during treatment from the dorsal side, was 5.3 $\pm$ 1.4°C and 0.5 $\pm$ 0.5°C for the laser treatments and controls, respectively.

In seven of the nine horses that received laser treatment, the temperatures did not return to the respective pre-treatment baseline at 5 min after treatment.
The temperature response in the subcutis is illustrated in Fig. 2b. The increase in temperature was significantly higher during the total laser treatment compared with the controls. The average increase measured during treatment from the dorsal aspect was $5.7 \pm 1.0 \degree\text{C}$ and $1.8 \pm 0.7 \degree\text{C}$ for laser treatments and controls, respectively. In all horses that received laser treatment, the temperature did not return to the respective pre-treatment baseline at 5 min after the treatment.

The temperature in the fetlock joint increased during both laser treatment and in the controls ($1.8 \pm 0.4 \degree\text{C}$ and $2.9 \pm 0.7 \degree\text{C}$, respectively) (Fig. 2c). No significant differences in temperature were observed between irradiated and control joints.

**Table 1** Mean ± standard error of temperature variables ($\degree\text{C}$) in standing horses during laser treatment. The treatment was applied to three projections: lateral, dorsal and medial. Total treatment represents the mean value of the whole treatment period.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Pre-treatment</th>
<th>Lateral projection</th>
<th>Dorsal projection</th>
<th>Medial projection</th>
<th>Total treatment</th>
<th>Post-treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skin ($n = 9$)</td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>Laser</td>
<td>29.5 ± 1.6</td>
<td>30.7 ± 1.6</td>
<td>34.8 ± 1.5</td>
<td>32.5 ± 0.9</td>
<td>32.7 ± 1.4*</td>
<td>31.9 ± 1.1</td>
</tr>
<tr>
<td>Control</td>
<td>29.8 ± 1.1</td>
<td>30.3 ± 1.4</td>
<td>30.3 ± 1.5</td>
<td>30.3 ± 1.6</td>
<td>30.3 ± 1.4</td>
<td>30.5 ± 1.6</td>
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<td>Subcutis ($n = 10$)</td>
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</tr>
<tr>
<td>Laser</td>
<td>30.3 ± 1.4</td>
<td>31.7 ± 1.2</td>
<td>36.0 ± 0.9</td>
<td>35.9 ± 0.2*</td>
<td>34.6 ± 1.1*</td>
<td>35.6 ± 0.1</td>
</tr>
<tr>
<td>Control</td>
<td>31.9 ± 0.9</td>
<td>33.3 ± 0.6</td>
<td>33.7 ± 0.5</td>
<td>33.9 ± 0.4</td>
<td>33.6 ± 0.6</td>
<td>34.0 ± 0.5</td>
</tr>
<tr>
<td>Fetlock joint ($n = 9$)</td>
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<tr>
<td>Laser</td>
<td>33.3 ± 1.0</td>
<td>34.3 ± 0.9</td>
<td>35.1 ± 0.7</td>
<td>35.7 ± 0.6</td>
<td>35.0 ± 0.7</td>
<td>36.0 ± 0.5</td>
</tr>
<tr>
<td>Control</td>
<td>29.9 ± 1.4</td>
<td>31.6 ± 1.0</td>
<td>32.8 ± 0.8</td>
<td>33.7 ± 0.6</td>
<td>32.7 ± 0.9</td>
<td>34.3 ± 0.5</td>
</tr>
</tbody>
</table>

*Significantly different from control group ($P < 0.05$).

FIG.1 Placement of the temperature probes to the (a) skin, (b) subcutis and (c) fetlock joint

FIG.2 Temperature in standing horses during laser treatment: (a) skin, $n = 9$; (b) subcutis, $n = 10$; (c) intra-articular, $n = 9$. The treatment was applied to three projections: Lateral, dorsal and medial. Values are expressed as mean ± SE for the two groups, laser and control. *Significantly different from control group ($P < 0.05$)
The temperature did not return to the baseline during the experimental session.

**Anaesthetized horses**

The temperature response is shown in Table 2. The results presented for the skin temperature are based on data from seven treated horses and four controls. The temperature response in the skin is shown in Fig. 5a. The increase in temperature was significantly higher during the laser treatment compared with the controls (3.2 ± 0.7°C and 0.0 ± 0.2°C, respectively). There was a significant difference in temperature during the 5-min period after treatment (2.2 ± 0.5°C and 0.3 ± 0.0°C, respectively). In five of the seven horses that received laser treatment, the temperature had not returned to the respective pre-treatment baseline at 30 min after treatment.

The temperature response in the subcutis is summarized in Fig. 3b. The increase in temperature was significantly higher during the laser treatment than in the controls (5.5 ± 1.4°C and -0.2 ± 0.4°C, respectively). There was a significant difference in temperature during both the 5-min period (2.9 ± 0.8°C and 0.1 ± 0.3°C, respectively) and the 10-min period (1.7 ± 0.5°C and 0.3 ± 0.1°C, respectively) after treatment. In seven of the eight horses that received laser treatment, the temperature had not returned to the respective pre-treatment baseline at 30 min after treatment.

The temperature in the fetlock joint was unaffected in both laser-treated and control groups (0.1 ± 0.0°C and 0.1 ± 0.0°C for the laser-treated and control groups, respectively). In addition, the temperature in the fetlock joint was unaffected in both laser-treated and control groups (0.1 ± 0.0°C and 0.0 ± 0.1°C, respectively) in the extended study. No significant differences in temperature were observed between the laser-treated and control groups.

**Discussion**

The present study shows significantly higher skin and subcutis temperatures after defocused CO₂ laser treatment compared with controls, in both standing and anaesthetized horses. However, no significant differences were observed in the fetlock joint temperature. To the best of our knowledge, no study has been performed on the thermal effects of defocused CO₂ laser treatment in horses. Therefore, comparison can only be made with studies on other modalities with a thermal effect.

**Methodology**

The study was performed on standing horses to mimic a true treatment procedure, with all physiological regulatory systems intact. The insertion of the thermistor into the fetlock joint was made through a needle. Although this method is well documented, it is likely that the mechanical effect of the thermistor itself may have affected the joint temperature. Therefore, to avoid voluntary movement of the horse, one part of the study was performed on anaesthetized horses, taking into account the fact that general anaesthesia and positioning affect the temperature and perfusion.

**Table 2** Mean ± standard error of temperature variables (°C) in anaesthetized horses during laser treatment. The treatment was applied to the dorsal projection only.

<table>
<thead>
<tr>
<th></th>
<th>Pre-treatment</th>
<th>Treatment 0–5</th>
<th>Treatment 6–10</th>
<th>Treatment 11–15</th>
<th>Treatment 16–20</th>
<th>Treatment 21–25</th>
<th>Treatment 26–30</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Skin (n = 11)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Laser</td>
<td>30.2 ± 0.4</td>
<td>33.5 ± 0.9*</td>
<td>32.3 ± 0.5*</td>
<td>31.8 ± 0.5</td>
<td>31.7 ± 0.5</td>
<td>31.8 ± 0.6</td>
<td>31.8 ± 0.6</td>
</tr>
<tr>
<td>Control</td>
<td>30.3 ± 1.1</td>
<td>30.3 ± 1.2</td>
<td>30.7 ± 1.1</td>
<td>31.0 ± 1.1</td>
<td>31.2 ± 1.0</td>
<td>31.4 ± 1.0</td>
<td>31.5 ± 1.0</td>
</tr>
<tr>
<td><strong>Subcutis (n = 12)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Laser</td>
<td>31.9 ± 0.4</td>
<td>37.4 ± 1.4*</td>
<td>34.8 ± 0.6*</td>
<td>33.6 ± 0.5*</td>
<td>33.4 ± 0.5</td>
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<tr>
<td>Control</td>
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<td>31.7 ± 1.4</td>
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<td><strong>Fetlock joint (n = 12)</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Laser</td>
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<td>33.4 ± 0.6</td>
<td>33.7 ± 0.5</td>
<td>33.9 ± 0.6</td>
<td>33.9 ± 0.6</td>
<td>33.8 ± 0.7</td>
<td>33.8 ± 0.7</td>
</tr>
<tr>
<td>Control</td>
<td>34.1 ± 0.7</td>
<td>34.2 ± 0.8</td>
<td>34.4 ± 0.8</td>
<td>34.5 ± 0.8</td>
<td>34.6 ± 0.7</td>
<td>34.7 ± 0.7</td>
<td>34.8 ± 0.7</td>
</tr>
<tr>
<td><strong>Extended study</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Laser</td>
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<td>29.9 ± 0.8</td>
<td>29.9 ± 0.7</td>
<td>29.9 ± 0.6</td>
<td>30.0 ± 0.6</td>
<td>30.0 ± 0.5</td>
<td>30.0 ± 0.4</td>
</tr>
<tr>
<td>Control</td>
<td>29.4 ± 0.4</td>
<td>29.4 ± 0.4</td>
<td>29.4 ± 0.4</td>
<td>29.8 ± 0.4</td>
<td>29.7 ± 0.3</td>
<td>29.9 ± 0.3</td>
<td>30.1 ± 0.4</td>
</tr>
</tbody>
</table>

*Significantly different from control group (P < 0.05).
An earlier study demonstrated that the guiding light (HeNe source) has no thermal effect\(^2\). Consequently, the influence of the guiding light used in this study was disregarded.

It has been reported that the surface temperature of the distal limb in horses differs between individuals\(^2\) and it is known that ambient temperature has an influence on skin temperature.\(^2\) This variation was greater at an ambient temperature of \(5^\circ\text{C}\) than at higher temperatures (15–25\(^\circ\text{C}\)). In the present study, the temperature varied between approximately 16 and 20\(^\circ\text{C}\).

**Temperature**

The increase in temperature in the skin was acute and of short duration, while the rise in temperature in the subcutis was more prolonged. This could be explained by the different thermal properties of the two tissue types caused by variations in the content of fat, protein and water, as well as tissue density and thermal conductivity.\(^2\)

The peak temperatures recorded during the treatment from the dorsal projection are due to the close position to the temperature probes located at the dorsal side of the fetlock joint. This also indicates that the accumulation of energy is local. However, some accumulation of heat probably occurs since the temperature of the last treatment projection, i.e. the medial projection, was higher than the first, lateral projection.

Despite the differences in the treatment protocol for the standing and anaesthetized groups, a longer accumulation of heat was observed in both skin and subcutis in the anaesthetized group compared with the standing group of horses. However, no statistical difference was seen between the treatment and after-treatment period in the standing group (Table 1).

![Figure 3](image1.png)  
**FIG. 3** Temperature in anaesthetized horses during laser treatment: (a) skin, \(n = 11\); (b) subcutis, \(n = 12\). The treatment was applied to the dorsal projection. Values are expressed as mean \(\pm\) SE for the two groups, laser and control. *Significantly different from control group \((P < 0.05)\)*

![Figure 4](image2.png)  
**FIG. 4** Temperature in clipped and unclipped, anaesthetized horses during laser treatment: (a) skin, \(n = 5\); (b) subcutis, \(n = 6\). Values are expressed as mean \(\pm\) SE for the two groups, laser and control. *Significantly different from control group \((P < 0.05)\)*

<table>
<thead>
<tr>
<th>Table 3</th>
<th>Mean (\pm) standard error of temperature variables ((^\circ\text{C})) in clipped and unclipped, anaesthetized horses during laser treatment. The treatment was applied to the dorsal projection only</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre-treatment</td>
</tr>
<tr>
<td>Skin ((n = 5))</td>
<td></td>
</tr>
<tr>
<td>Clipped</td>
<td>29.0 (\pm) 0.4</td>
</tr>
<tr>
<td>Unclipped</td>
<td>29.7 (\pm) 1.1</td>
</tr>
<tr>
<td>Subcutis ((n = 6))</td>
<td></td>
</tr>
<tr>
<td>Clipped</td>
<td>32.9 (\pm) 0.6</td>
</tr>
<tr>
<td>Unclipped</td>
<td>33.0 (\pm) 0.7</td>
</tr>
</tbody>
</table>

*Significantly different from unclipped group \((P < 0.05)\).*
Effect of defocused CO₂ laser on equine temperature

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compared with a statistical difference in skin (5 min) and subcutis (10 min) in the anaesthetized group (Table 2). It is likely that the anaesthetized horses were affected, to some extent, by the differences in tissue perfusion due to the position of the limb and the decreased magnitude of blood flow during anaesthesia. It is also known that both halothane and isoflurane induce peripheral vasodilatation and decreased cardiac output. These inhalant anaesthetics override the physiological effects of individual induction agents early during anaesthesia. Thus, the circulatory effects during anaesthesia may alter the potential for heat dissipation in peripheral tissue.

In the standing group, most standard errors in the pre-treatment periods were greater than in the post-treatment periods. The most likely explanation is that a physiological regulatory mechanism causes an increase in temperature during treatment. It is reported that when tissue temperature reaches a certain level, it triggers physiological responses such as blood flow, and a stabilization of temperature may occur. An alternative explanation is a micro trauma caused by insertion of the thermistor. The initial trauma may lead to an increase in temperature due to minor bleeding. The differences in temperature standard error were smaller in the anaesthetized groups, which support these suggestions.

The results of the present study correspond well with those from studies on other modalities with thermal effects. For example, a gel wrap heated to 40°C and applied to the metacarpal region for 30 min raised skin temperature by 5°C and applied to the metacarpal region for 30 min raised skin temperature by 5°C. Notably, in most of the treated horses, mild or moderate oedema was seen in the treated leg at the end of the study. In another study, therapeutic ultrasound caused a significant increase of less than 1°C between the treated and untreated leg in horses and an increase of approximately 2-5°C in canine thigh muscle. The results of the present study conform to these findings, indicating that the treatment was well tolerated and pain-free.

Clinical relevance

In the present study, the laser treatment was well tolerated and, in accordance with earlier studies, a moderate to vigorous heating effect was achieved in the skin and subcutis. In designing a rehabilitation programme, it is of importance to select a thermal modality that elevates the temperature to a suitable level at the site of treatment. According to Oostervald and Rasker, it is unsuitable to use modalities that elevate intra-articular temperature when treating active arthritis or secondary synovitis in osteoarthritis. It is likely that a higher laser dose may result in higher superficial tissue temperatures, but also a possibly unwanted increase of the intra-articular temperature, as well as a higher risk for potential damage to the skin. It is of great importance that the horse is able to perceive pain as a warning that injury threshold levels are exceeded, and that steps should be taken to minimize the effects of repeated exposures. This raises the question of the suitability of sedation of horses during treatment. Since commonly used α₂-agonists produce both significant sedation and analgesia, use of these drugs may reduce the response to heat and pain. Notably, with the protocol used in this study, there was a minimal risk of thermal injury.
Conclusion

The aim of the present randomized, double-blind study was to investigate if defocused CO2 laser treatment (71–171 cm−2) affected the temperature of the skin, subcutis and fetlock joint in standing and anesthetized horses. Treatment with defocused CO2 laser on the fetlock joint region mediated a significant heating affect in the skin and subcutis, but no differences in fetlock joint temperature were observed. However, it was not shown whether the increase in surface temperature reflects changes in local perfusion, mediates pain relief or restores function. Further research is therefore necessary to describe the physiological and clinical effects of defocused CO2 laser treatment.

Acknowledgements

The authors are grateful to The Swedish Racing and Totalizator Board (ATG) for financing the study, and to EL.EN. Srl, Italy for supplying the laser system. The authors thank Karin Thulin, Kristina Karlström, Martina Andersson and Anna Edner for technical assistance.

References

Effect of defocused CO₂ laser on equine temperature


Effect of Defocused CO₂ Laser on Equine Tissue Perfusion

By A. Bergh¹, G. Nyman², T. Lundeberg³ and S. Drevemo¹

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Introduction

The goal of physical therapy is to promote healing of tissues through stimulation of normal physical processes, thereby restoring the function of the injured tissue (Stashak 1987). Therapeutic modalities in the form of hot packs, therapeutic ultrasound, and lasers have been advocated by numerous practitioners working with sports injuries, both in humans and horses (Lehmann 1990, Bromiley 1991). Simplified, laser therapy can be divided into surgical lasers (i.e. high-effect laser) and lasers used for biomodulation, i.e. low level laser therapy (often treatment dosages of <1 to 4 J/cm² on treatment sites) (Basford 1995). However, lasers originally made for surgery are used as biomodulating lasers; with a defocused beam and at a lower output effect, but with doses higher than in low laser therapy. A recent prospective study indicates that, defocused carbon dioxide (CO₂) laser may be an applicable treatment for acute synovitis in horses (Lindholm et al. 2002). The effects of laser radiation on tissue structure and function are, however, unclear. Laser therapy is proposed to produce photochemical effects by excitation of electronic states in molecules and chromophores. Unlike low level laser, CO₂ laser is also proposed to have photothermal effects, by transformation of absorbed light en-
ergy to heat (Thomson 1991). The light is absorbed at a depth of less than 0.1 mm (Bhatta 1994), which indicates a possible heating effect in superficial tissues.

Results from our own studies show that treatment with defocused CO₂ laser causes a significant increase in the temperature of skin and subcutis (Bergh et al. 2005). A rise in local temperature generally correlates with an increase in perfusion, and is believed to have a positive effect on pain and tissue regeneration (Lehmann 1990, Nannemann 1991, Oosterveld and Rasker 1994, Wright and Sluka 2001, Nadler et al. 2002). Vasodilatation increases blood flow to reduce ischemia of injured tissue, resulting in decreased activity of the pain receptors. A greater blood flow increases the supply of nutrients to the area for the repair process and removes by-products from the injured tissue. To the best of our knowledge, there are no studies published on the effect of defocused CO₂ laser on equine tissue perfusion.

Laser Doppler Flowmetry (LDF) technique is widely used for measurement of tissue perfusion (Norman et al. 1992, Adair et al. 1994, Raisis et al. 2000a, Berardesca et al. 2002, Edner et al. 2002, McGorum et al. 2002). This technique provides a continuous measure of relative perfusion, allowing detection of changes in blood flow over time on a single site (Raisis et al. 2000b, Raisis et al. 2000c, Humeau et al. 2004). In the present study, a hypothesis was formulated that treatment with defocused CO₂ laser increases temperature and perfusion in skin and underlying muscle. The objective was to measure temperature and perfusion in anaesthetized horses treated with active or sham laser. A further aim was to compare the effect of laser treatment on clipped and unclipped skin.

Materials and Methods

Horses

The study comprised ten, healthy Standardbred trotters (6 females and 4 geldings) with a mean weight of 497 kg (range 411-578 kg) and a mean age of nine years (range 4-19 years). Eight horses received laser treatment and two served as controls. All horses were pigmented at the irradiated area. The Ethical Committee on Animal Experiments in Uppsala, Sweden has approved the study.

Anaesthetic protocol

Food was withheld for 12 hours prior to anaesthesia, but water was available until premedication. The horses were premedicated with detomidin (Domosedan® vet; Orion Pharma AB, Sollentuna, Sweden). Anaesthesia was induced intravenously with guaifenesin (Myolaxin® vet. diluted to 7.5%; Chassot & Cie AG, Berne, Switzerland) and tiopentone (Pentothal Sodium 12.5%; Abbott, Solna, Sweden). The horses were intubated, transported to the surgical table and placed in dorsal recumbency. Anaesthesia was maintained with isoflurane (Forene; Abbott, Solna, Sweden) in oxygen. Electrolytes (Ringer acetate; Pharmacia & Upjohn, Stockholm, Sweden) were infused through a catheter in the jugular vein. Spontaneous breathing was allowed from a semiclose, large-animal circle. To detect any changes in depth of anaesthesia, arterial blood pressure as well as heart rate was monitored throughout the research protocol.

Peripheral perfusion and muscle temperature

Laser Doppler Flowmetry (LDF) was performed using a Periflux 4001 flowmeter (Perimed, Järfläla, Sweden). A treatment area of 6x7 cm on each semimembranous muscle was prepared; one side clipped and the other with the coat intact. A small area for the measuring probes was prepared, in direct contact with each
treated area. Skin perfusion was measured on the skin surface (Probe 407, Perimed, Järfälla, Sweden), 1 and 3 cm from the treated area. For muscle perfusion, a straight microtip with slanted tip (MT A500-0.120 mm, 0.5 mm diameter, Perimed, Järfälla, Sweden) was placed in the semimembranosus muscle of the right and left hind limb, close to the skin perfusion probe at 1 cm from the treatment area. The microtip was inserted via a 0.7 mm cannula to a depth of 3 cm and connected to a probe (Master Probe; Probe 418-x, Perimed, Järfälla, Sweden), after which the cannula was retracted. Skin and muscle temperatures were measured using thermistor probes (skin-440, muscle-442-PI, Perimed, Järfälla, Sweden) connected to a recording unit (PF 5020, Perimed, Järfälla, Sweden). The temperature probes were attached to the skin or inserted in the muscle approximately 1 cm from its corresponding perfusion probe and at the same distance from the irradiated area. Flux, expressed in blood perfusion units, and temperature, were displayed and recorded continuously (Perisoft 1; 14, Perimed, Järfälla, Sweden). The total recording time was 50 min on average. To allow comparison of results, the LDF probes were calibrated in a standard motility solution provided by the manufacturer. The following skin and muscle blood flow and temperature features were analysed:

1. The average value before treatment, sampled for one minute (baseline).
2. The average value during treatment, sampled for one minute (treatment).
3. The peak value during treatment (peak).
4. The time from start of treatment to the peak value, in seconds (time to peak).

**Laser protocol**

A defocused CO₂ laser (10 600 nm, KSV 25S; EL.EN. SRL, Firenze, Italy) was used in the study, see Table 1. As guiding light, a HeNe source (633 nm) emitted continuously at 1.2 mW. The laser system was calibrated regularly, and an external detector (LaserMate Detector, COA-33-0191-000; Gamma Optronic AB, Uppsala, Sweden) was used to measure the intensity of the laser beam before and after each treatment. The laser system was set to give continuous output power of 16 W at a distance of 1 m, during a treatment period of 4 min. The treatment area was 42 cm² (6x7 cm) and the irradiation energy 91 J/cm². Laser-treated and control horses, as well as the order of the treatment to the clipped and unclipped area, were randomized. There was an average of a 60-min pause between the irradiation of the clipped and unclipped area.

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**Table 1. Laser parameters, dosage, and mode of application (KSV 25S Laser device)**

<table>
<thead>
<tr>
<th>Lasing media</th>
<th>CO₂</th>
<th>HeNe</th>
</tr>
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<tbody>
<tr>
<td>Wavelength</td>
<td>10 600 nm</td>
<td>633 nm</td>
</tr>
<tr>
<td>Continuous output power</td>
<td>16 W</td>
<td>0.0012 W</td>
</tr>
<tr>
<td>Mode of application</td>
<td>scanning</td>
<td>scanning</td>
</tr>
<tr>
<td>Diameter of beam at source</td>
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</tr>
<tr>
<td>Divergence</td>
<td>1.5 mrad</td>
<td>2.0 mrad</td>
</tr>
<tr>
<td>Treatment distance</td>
<td>100 cm</td>
<td>100 cm</td>
</tr>
<tr>
<td>Irradiated area</td>
<td>42 cm²</td>
<td>42 cm²</td>
</tr>
<tr>
<td>Site of application</td>
<td>semimembranosus muscle</td>
<td>semimembranosus muscle</td>
</tr>
<tr>
<td>Treatment time</td>
<td>4 min</td>
<td>4 min</td>
</tr>
<tr>
<td>Dosage at skin surface</td>
<td>91 J/cm²</td>
<td>0.007 J/cm²</td>
</tr>
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</table>
Statistical analysis
Statistica 6.0 (Statsoft, 2001; Statsoft Scandinavia AB, Uppsala, Sweden) was used for data analysis, and results are presented as means and standard errors (SE). Microvascular perfusion and temperature were calculated as the average of one minute of stable recordings; immediately before start of treatment, at the end of treatment, and at 4 min after the end of treatment. The perfusion data are presented as relative changes in perfusion, using arbitrary perfusion units (PU). The data were individually corrected by setting the baseline before treatment to 100%. Biological zero (i.e. the laser Doppler signal from non-perfused tissue) was not subtracted; however, perfusion values under 3.5 PU were excluded from the analysis as the biological zero for equines is approximately 1.6 PU for skin and 3.5 PU for muscle (unpublished results).

Treatment data were compared to baseline data within each group. Statistical calculations comparing the time to maximum (peak) values and peak values for both skin temperature and perfusion were also made. Statistical calculations were performed with Wilcoxon signed rank test and Mann-Whitney test, when appropriate. Statistical significance was accepted at p<0.05.

Results
None or only minor differences in arterial blood pressure or heart rate were found within each protocol or between treatment and control.

The results are presented separately for treated and control horses. Figure 1 shows representative temperature and perfusion curves from one laser and control recording, respectively. As demonstrated by the laser recording, the increase in temperature is almost immediately followed by an increase in perfusion.

The laser- treated group
Temperature
The temperature response to laser treatment is presented in Table 2. The results presented for temperatures response of clipped skin, 1 cm and unclipped skin, 3 cm are from seven horses, as measurements from one horse had to be excluded due to technical problems. There was a significant increase in temperature in all skin

<table>
<thead>
<tr>
<th>Area</th>
<th>Baseline</th>
<th>Treatment</th>
<th>Difference</th>
<th>Peak value</th>
<th>Time to peak</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>º C</td>
<td>º C</td>
<td>º C</td>
<td>º C</td>
<td>s</td>
</tr>
<tr>
<td>Muscle</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>clipped (n=8)</td>
<td>32.8±1.3</td>
<td>34.0±0.7</td>
<td>1.2±0.7</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>unclipped (n=8)</td>
<td>34.0±0.5</td>
<td>34.4±0.3</td>
<td>0.4±0.3</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
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<td>Skin 1 cm</td>
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<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>clipped (n=7)</td>
<td>30.4±1.0</td>
<td>35.9±0.8*</td>
<td>5.5±1.5</td>
<td>36.9±1.4</td>
<td>175.7±28.8</td>
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<td>unclipped (n=8)</td>
<td>29.7±1.3</td>
<td>34.5±1.6*</td>
<td>4.8±1.4†</td>
<td>35.1±1.9</td>
<td>164.0±18.8†</td>
</tr>
<tr>
<td>Skin 3 cm</td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>clipped (n=8)</td>
<td>31.5±1.0</td>
<td>37.0±1.0*</td>
<td>5.5±1.5</td>
<td>37.0±1.3</td>
<td>169.6±16.4†</td>
</tr>
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<td>unclipped (n=7)</td>
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<td>34.7±1.0*</td>
<td>2.1±0.4</td>
<td>35.6±1.1</td>
<td>230.0±21.9</td>
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</tbody>
</table>

Values are presented as means ±SE; measured as temperature (º C); time to peak (s), n.d. = not detected.* Significantly different from baseline, † significantly different from unclipped skin 3 cm from irradiated area, p<0.05.
recordings, i.e. 1 cm and 3 cm from the irradiated area, compared to the respective baseline recordings, for both clipped and unclipped hair coat. The temperatures did not return to the pre-treatment baseline at 30 min after treatment in: 2/7 of the horses in the skin 1 cm clipped group, 2/8 in the skin 1 cm unclipped group, 5/8 in the skin 3 cm clipped group, and 2/7 in the 3 cm unclipped group.

No significant difference was recorded in muscle temperature for either clipped or unclipped hair coat. The time of peak response to laser treatment is presented in Table 2. Peak temperature occurred earlier in clipped than in unclipped skin measured 3 cm from the irradiated area. In the unclipped groups, peak temperature occurred later in the 3 cm than in the 1 cm recording point.

Perfusion

The perfusion response to laser treatment is presented in Table 3. The results presented for muscle perfusion are from seven (clipped skin) and six (unclipped skin) horses, since values less than 3.5 PU were excluded from statistical analysis. There was a significant increase in perfusion in all skin recordings, i.e. 1 and 3 cm from the irradiated area for both clipped and unclipped hair coat. The perfusion did not return to the pre-treatment baseline 30 min after treatment in: 3/8 of the horses in the 1 cm clipped group, 1/7 in the 1 cm unclipped group, 2/8 in the 3 cm clipped group, and 2/7 in the 3 cm unclipped group. There was no significant difference in muscle perfusion.

The time for peak response to laser treatment is presented in Table 3. There was no significant difference in time to peak.

The control group

Temperature and perfusion data are presented in Table 4. None or only minor changes were seen in tissue temperature or tissue perfusion in the two horses used as controls.

Discussion

In the present study, there was an increase in temperature and perfusion in skin, but not in the underlying muscle. Two major findings were identified: (1) treatment with defocused CO₂
laser causes an increase in temperature of the skin in clipped and unclipped haircoat, 1 and 3 cm from the irradiated area; (2) the increase in temperature was accompanied by an increased perfusion. To the best of our knowledge, no study has been performed on the effects on blood perfusion of defocused CO\textsubscript{2} laser treatment in horses. Therefore, comparisons can only be made with studies on other modalities with an effect on tissue temperature and/or blood perfusion. Studies on acupuncture, transcutaneous electric nerve stimulation, superficial heat and continuous therapeutic ultrasound have all shown increase in temperature and/or perfusion (Nannemann 1991, Oosterveld and Rasker 1994, Cramp et al. 2000, Levine et al. 2001, Wright and Sluka 2001, Kuo et al. 2004). Therapeutic application of heat plays a major role in rehabilitation programs. The rationale for using different heating modalities is based primarily on the fact that they produce peak temperatures in different locations. The goal is to achieve a “therapeutic” level of temperature elevation without causing adverse responses.

As one of the explanations for the mode of action of defocused CO\textsubscript{2} is its photothermal effect, it is important to identify the heating pattern of laser treatment. In the present study, the increases in temperature and perfusion were in superficial tissues, and not in muscle temperature and blood perfusion. As in other superficial heating modalities, the deeper tissues including muscles are usually not significantly heated. Heat transfer from the skin surface into deeper tissues is inhibited by the subcutaneous fat, which acts as a thermal insulator, and by the increased blood flow in more superficial tissues which cools the tissues by transporting away the heat (Lehmann 1990).

The physiological effect of the applied CO\textsubscript{2} laser irradiation is related to the activation of warmth and heat receptors and afferents; cutaneous thermosensitive A\textdelta and the C-fibres (Arendt-Nielsen and Chen 2003). In the present study, it is likely that both A\textdelta and C-fibres were stimulated, with a secondary influence on blood perfusion. The mechanism for vasodilatation is suggested to be activation of the axon/dorsal

<table>
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<tr>
<th>Area</th>
<th>Baseline Temperature</th>
<th>Treatment Temperature</th>
<th>Baseline Perfusion</th>
<th>Treatment Perfusion</th>
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<tr>
<td></td>
<td>° C</td>
<td>° C</td>
<td>%</td>
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</tr>
<tr>
<td>Muscle</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>clipped</td>
<td>34.5 (34.4-34.6)</td>
<td>34.5 (34.4-34.6)</td>
<td>100</td>
<td>102.5 (99-106)</td>
</tr>
<tr>
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<td>34.4 (33.8-35.0)</td>
<td>34.4 (33.7-35.0)</td>
<td>100</td>
<td>98.0 (98)</td>
</tr>
<tr>
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<td></td>
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<tr>
<td>clipped</td>
<td>29.1 (25.2-33.0)</td>
<td>28.9 (24.9-32.9)</td>
<td>100</td>
<td>93.0 (86-100)</td>
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<td>unclipped</td>
<td>26.7 (26.5-26.9)</td>
<td>26.6 (26.2-27.0)</td>
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<td>96.0 (96)</td>
</tr>
<tr>
<td>Skin 3 cm</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>clipped</td>
<td>32.8 (32.8-32.9)</td>
<td>32.7 (32.5-32.9)</td>
<td>100</td>
<td>102.5 (94-111)</td>
</tr>
<tr>
<td>unclipped</td>
<td>32.9 (32.2-33.6)</td>
<td>32.8 (32.2-33.4)</td>
<td>100</td>
<td>79.0 (76-82)</td>
</tr>
</tbody>
</table>

Values are presented as means/medians and ranges; measured as temperature (° C); perfusion (PU) with the baseline set to 100%, n=2.
root ganglion reflex from heat sensitive nociceptive afferents, which releases neurotransmitters that increase blood flow. These neurotransmitters may stimulate nitric oxide release causing further vasodilatation (Kellogg et al. 1999, Minson et al. 2001, Stephens et al. 2001). Thermally evoked vasodilatation has also been found following non-painful stimulation when using a slowly increasing heat stimulus (Magerl and Treede 1996, Minson et al. 2001). In the present study, the perfusion at 1 cm from the irradiated area increased with 146 PU on average, when the temperature had increased by about 6 °C, to a mean of approximately 36 °C. This is consistent with results from other studies that report on significant vasodilatation between local temperatures of 30-35 °C (Barcroft and Edholm 1943, Taylor et al. 1984, Johnson et al. 1986, Stephens et al. 2001). In humans, local warming of the skin to 42°C has been reported to increase blood flow tenfold, at the end of a 20-min warming period (Saumet et al. 1998).

The increase in perfusion started directly after the first rise in temperature. This is in agreement with an earlier study showing a correlation between the first sensation of non-noxious heat and the onset of cutaneous vasodilatation, and that the vasodilatation correlates better with the sensation of heat compared to actual skin temperature (Stephens et al. 2001). Our findings, and the fact that vasodilatation was detected 3 cm from the irradiated site, support the suggestion that the observed vasodilatation was caused by an axon/dorsal root ganglion reflex of nociceptive afferents, probably in combination with a secondary release of nitric oxide (Kellogg et al. 1999, Minson et al. 2001). There were no significant differences between the temperatures of clipped and unclipped skin. These results do not agree with earlier studies in which the skin temperature was higher in animals with long haircoat, compared to short or clipped hair (Steiss and Adams 1999, Bergh et al. 2005). However, it is possible that the relatively thin haircoat at the actual experimental site had an influence on the results. As the irra-

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Fig. 1. Representative tracing from one laser treated and one control horse, displaying temperature and perfusion response. The perfusion is presented as arbitrary Perfusion Units (PU) and temperature as °C. Channel 1; perfusion in muscle. Channel 2; perfusion in skin at 1 cm from the irradiated area. Channel 3; perfusion in skin at 3 cm from the irradiated area. Channel 4; temperature in muscle. Channel 5; temperature in skin 1 cm from the irradiated area. Channel 6; temperature in skin at 3 cm from the irradiated area.

A; one minute tracing immediately before the start of the treatment. B; one minute tracing at the end of the treatment. C; one minute tracing at four minutes after end of the treatment.
T; start of laser and sham treatment, respectively.

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Rations of the clipped and unclipped areas were randomized, it is unlikely that a consensual effect of the irradiation would have a major influence on the results.

Movement artefacts are a common problem using Laser Doppler Flowmetry technique. This was reduced as the horses were anaesthetised during the study. It is possible that tissue perfusion was affected by the anaesthetic agent and to some extent, by the position of the limb. In order to minimize the negative effects on peripheral perfusion, the anaesthesia was maintained with isoflurane, since hind-limb blood flow has been found to be higher during isoflurane than halothane anaesthesia, due to a less cardiac depression and greater peripheral vascular dilatation (Raisis et al. 2000a). Blood flow to a region is influenced by its vertical position relative to the heart (Hennig et al. 1995). This positional effect was minimized in our study since the position of the probes was horizontal and approximately at the level of the heart. However, it is likely, due to the influence of general anaesthesia and positioning of the limb, that the registered increases in blood perfusion in the anaesthetized horses were similar or less pronounced than would be expected in non-anaesthetized animals.

It has been reported that the surface temperature of the distal limb differs between individuals (Kameya and Yamaoka 1968, Webbon 1978, Palmer 1983) and it is known that the ambient temperature has an influence on skin temperature (Kameya and Yamaoka 1968, Webbon 1978). This variation was greater at an ambient temperature of 5 °C than at higher temperatures (15-25 °C) (Kameya and Yamaoka 1968, Palmer 1983). In the present study, the ambient temperature varied between approximately 16 and 20 °C.

In our study, defocused CO₂ laser radiation increased temperature and tissue perfusion in the skin, but not in deeper tissues. However, the question as to whether this has therapeutic significance remains to be investigated. The biophysical effects of similar temperature elevation in human body tissue include increased local blood flow and metabolism, elevated pain threshold, decreased muscle spindle firing rate, and increased extensibility of connective tissue. Heat can provide analgesia, promote relaxation, reduce muscle spasm, and enhance flexibility of muscles and periarticular structures (Lehmann 1990, Nannemann 1991, Minor and Sanford 1993, Wright and Sluka 2001). Heat also assists in resolution of inflammatory infiltrates, oedema and exudates (Lehmann 1990, Nannemann 1991). Consequently, the increase in temperature and perfusion in the present study may have had an effect on pain and tissue regeneration.

In conclusion, defocused CO₂ laser causes a significant increase in skin perfusion, which is correlated to the increase in skin temperature, both measured at 3 cm from the irradiated area. No differences were observed between clipped and unclipped haircoat, or in muscle. Further studies are needed to investigate if the increase in temperature and perfusion achieved by defocused CO₂ laser enhances tissue regeneration, decreases pain and restores impaired function.

Acknowledgements
This project was supported by grants from the Swedish Racing and Totalizator Board (ATG), and EL.EN. Srl, Italy supplied the laser system. The authors express their sincere gratitude to Karin Thulin, Annelie Rydén, Pia Funkquist, Anna Edner, Birgitta Essén-Gustavsson for technical assistance, and Patrik Öhagen for statistical consultation. Many thanks also to Björn Bakken and the late Bertil Gazelius at Perimed for excellent help with the laser Doppler system.

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**Sammanfattning**

Lokala blodflödesförändringar hos häst vid behandling med defokuserad CO₂ laser. Laserbehandling sägs stimulera och påskynda ned-sänktsprocessen, men dess verkningsmekanism är oklar. En nyligen publicerad studie visar att behandling med defokuserad CO₂ laser ger en ökning av temperaturen i yttiga vävnader hos häst. En ökning av vävnadstemperatur åtföljs ofta av en ökning av det lokala blodflödet, med en positiv inverkan på vävnadens läkning. Så vitt vi vet saknas publicerade studier om defokuserad CO₂ lasers effekt på blodflöde hos häst. Syftet med denna studie var att undersöka effekten av defokuserad CO₂ laser på lokalt blodflöde (med hjälp av Laser Doppler Flowmetry) och att korrelera blodflödet till temperaturen i rakad och orakad hud, samt i underliggande muskelvävnad. Ti hästar inkluderades i studien, varav åtta fick aktiv laser och två placebo. Den aktiva laserdosen var 91 J/cm² och gavs på ett 42 cm² stort område över semimembranosus muskulaturen. Den aktiva laserbehandlingen ökade signifikant blodflöde och temperatur, med i genomsnitt 146.3±33.4 perfusionsenheter (334%) och 5.5±1.5 °C i rakad hud, och 80.6±20.4 perfusionsenheter (264%) och 4.8±1.4 °C i orakad hud. Inga statistiskt signifikanta skillnader kunde noteras i blodflöde och temperatur i underliggande muskel, eller mellan rakad och orakad hud. Fortsatta studier får visa om denna temperatur- och blodflödesökning kan leda till smärtlindring och förbättrad läkning.

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Defocused CO₂ laser on equine skin: A histological examination

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Keywords: defocused CO₂ laser; equine, histology; skin; thermal injury

Summary

Reasons for performing study: Lasers originally made for surgery, such as carbon dioxide (CO₂) laser, are today used as biomodulating lasers; with a defocused beam and at submaximal output effect, but with doses and a thermal effect that is higher than used in low-level laser therapy. No studies have, however, been published on effects of this treatment on equine skin histology. A better understanding of this will help us define how lasers should be used, in order to reduce potential side effects.

Objective: The objective was to describe the acute effects of different doses of defocused CO₂ laser, ranging from therapeutic to surgical levels, on equine skin.

Methods: Defocused CO₂ laser was administered to the skin in the hamstrings (91 J/cm²), the fetlock (137 J/cm²), and the loin (450 J/cm²) areas of 13 Standardbred horses. The acute effects on skin histology (90 min after end of therapy) were examined.

Results: Mild changes with focal spongiosis and subepidermal clefts were found after the 91 J/cm² irradiation and more severe changes with diffuse subepidermal clefts after the 137 J/cm² dose. Necrosis of epidermis, dermis and adnexal structures, and significant thinning of the epidermis was observed after the 450 J/cm² dose.

Conclusions: The present study indicates acute dose-dependent changes in equine skin histology after laser irradiation. Severe tissue damage was induced using the 450 J/cm² dose.

Potential relevance: To reduce the potential side effects of defocused CO₂ laser irradiation, the laser parameters must be carefully evaluated. Caution should be taken if doses higher than 91 J/cm² (16 W, 4 min, and 42 cm²) are used in irradiation of equine skin.
Introduction

Laser therapy is widely used in the rehabilitation of equine injuries (Bromiley, 1991; Ramey & Basford, 2000; Sutton, 2003). Simplified, laser therapy can be divided into surgical lasers (i.e. high power laser) and lasers used for biomodulation, i.e. low-level laser therapy with treatment doses of 10-15 J/cm² for musculoskeletal injuries (Basford, 2000). However, lasers originally made for surgery, such as the carbon dioxide (CO₂) laser, are today used as biomodulating lasers, with a defocused beam and at submaximal output power, but with higher doses than in low-level laser therapy.

Although some controversy exists, the concept that laser irradiation alters cellular processes in a wavelength-dependent manner seems established (Ramey & Basford, 2000). Despite the postulated effects that lasers increase blood perfusion, modulate pain or decrease inflammation, the precise explanation models are incomplete. Unlike low-level laser, defocused CO₂ laser is shown to have photothermal effects (Bergh et al., 2005) with increases in temperature and blood flow in superficial equine tissues (Bergh et al., 2006). A high increase in temperature may cause tissue damage and high power lasers have been studied in equines with respect to effects on the histology of skin, endometrium, synovial membranes and cartilage (Palmer & McGill, 1992; Collier, 1993; Pullin et al., 1996; Leib et al., 2001; Doyle-Jones et al., 2002). Relatively little data is, however, available for the effect of biomodulating lasers (Kaneps et al., 1984; Gomez-Villamandos et al., 1995; Petersen et al., 1999).

The CO₂ laser emits infrared light, which is converted into heat and almost entirely absorbed within the outer 100 µm of tissue (Carruth & McKenzie, 1986; Hecht, 1992). The degree of photothermal effect is dependent on the distribution of light in the tissues and the tissue thermal properties, as well as the temperature and duration at a certain temperature (Thomsen, 1991). Therapeutic heat is suggested to modify the pain sensation (Lehmann, 1990) in accordance to the gate theory (Melzack & Wall, 1965), and to stimulate release of pro-inflammatory and pain mediators with secondary influence on vasodilatation (Magerl & Treede, 1996; Minson et al., 2001). An area with an inflammatory reaction may function as a “counterirritant”, based on the proposed theory of “diffuse noxious inhibitory control” by Le Bars (2002). According to the theory, peripheral noxious stimuli activate a central inhibitory network which in turn inhibits dorsal horn neurons, thus causing an analgesic effect outside the stimulated zone. However, in order to avoid negative side effects, it is of great importance that the intensity of the thermal therapy is adjusted to the tissue treated.

To our knowledge, no studies have been published on the effects of defocused CO₂ laser on equine skin histology. A better understanding of the effects involved will help to select laser parameters and reduce potential negative side effects. In the present study, a hypothesis was formulated that treatment with defocused CO₂ laser causes dose-dependent morphological changes in the skin. The aim was to describe the in vivo effects of three different doses of defocused CO₂ laser, ranging from therapeutic to surgical doses, on the histology of equine skin.
Materials and methods

Horses
This study was part of an investigation concerning temperature and blood flow in skin (Bergh et al., 2006) and comprised thirteen, clinically healthy Standardbred trotters (8 females and 5 geldings) with a mean weight of 486 kg (range 375-578 kg) and a mean age of 10 years (range 2-24 years). Food was withheld for 12 h prior to anaesthesia, but water was available until premedication. The study was approved by the Ethical Committee on Animal Experiments in Uppsala, Sweden.

Procedures

Anaesthetic protocol
Ten horses were premedicated with detomidin (Domosedan® vet)¹ and three horses horses with acepromazine (Plegicil vet)² and methadone (Metadon)³. Anaesthesia was induced intravenously with guaifenesin (Myolaxin® vet diluted to 7.5%)⁴ and thiopentone (PentotalNatrium 12.5%)⁵. The horses were intubated, transported to the surgical table and placed in dorsal or left lateral recumbency. The right non-dependent forelimb, which was used for treatment, was supported in a position perpendicular to the body axis. Anaesthesia was maintained with isoflurane (Forene)⁵ in oxygen for the first 10 horses and halothane (Fluotane)⁶ in oxygen for the remaining three. An electrolyte solution (Ringer acetate)³ was continuously infused through a catheter in the left jugular vein. Spontaneous breathing was allowed from a semi-closed, large-animal circle. The eight horses receiving the 137 and 450 J/cm² doses were kept anaesthetized to be used for surgical training and were subsequently euthanised with pentobarbitone sodium (Avlivningsvätska för djur, 100 mg/ml)⁷.

Laser protocol
A defocused CO₂ laser (KSV 25S, 10 600 nm)⁸ was used in the study. As guiding light, a HeNe source (633 nm) emitted continuously at 1.2 mW. The laser light was continuously emitted through a scanning device creating a quasi-continuous delivery of radiation as the 6-mm-diameter laser spot was scanned back and forth over the irradiation area. The laser system was calibrated regularly, and an external detector (LaserMate Detector, COA-33-0191-000)⁹ was used to measure the intensity of the laser beam before and after each treatment.

Three laser doses on three different areas, proximal hamstring (91J/cm²), the dorsal fetlock (137 J/cm²) and the loin (450 J/cm²), were analysed (Table 1). The areas were shaved before irradiation. Each horse served as its own control, thus skin biopsies were taken from both the irradiated and representative non-irradiated areas in each horse.
Table 1. An overview of laser protocols

<table>
<thead>
<tr>
<th>Treatment parameters</th>
<th>Hamstrings (n=4)</th>
<th>Fetlock joint (n=7)</th>
<th>Loin (n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Site of application</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Output power (W)</td>
<td>16</td>
<td>16</td>
<td>20</td>
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<tr>
<td>Mode of application</td>
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<td>scanning</td>
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<tr>
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<td>100</td>
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<td>Irradiated area (cm²)</td>
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<td>42</td>
<td>16</td>
</tr>
<tr>
<td>Treatment time (min)</td>
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<td>137</td>
<td>450</td>
</tr>
<tr>
<td>Energy density (J/cm²)</td>
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</table>

Preparation of tissue samples
A total of 42 skin specimens were taken with an 8-mm disposable biopsy punch (Miltex Instrument Company, INC.,) from the center of the laser treated and corresponding control sites, approximately 90 min after laser irradiation. The skin samples were divided into two parts along the longitudinal axis and immediately fixed in cacodylate-buffered 3% glutaraldehyde (pH 7.2). The specimens were rinsed in 0.067 M cacodylate buffer (pH 7.2), dehydrated in a graded series of ethanol, infiltrated and embedded in Historesin. Sections of 2-μm were cut and stained with haematoxylin-eosin (H&E).

Histological examination
The sections were examined with special reference to the appearance of epidermis, epidermis-dermis junction, adnexal structures (hair follicles, sebaceous and sweat glands), cell infiltrates and blood vessel appearance of dermis and without the examiners knowledge of the different treatments. The changes were grouped in four categories regarding the type and severity of lesions.
Furthermore, computer-assisted measurements of epidermal thickness (Easy Image Analysis System) were obtained on every fiftieth micrometer of all sections. The shortest distance between the dermal-epidermal junction and the innermost aspect of the stratum corneum were measured. Areas containing hair follicles or technical artefacts were avoided during the measurement procedure. Digital images of sections were taken with a Nikon Microphoto-FXA imaging system.

Statistical analysis
Statistica 6.0 (Statsoft, 2001) was used for data analysis, and the results are presented as means and standard deviation (sd). Statistical calculations were performed with Student paired and unpaired t-tests, when appropriate. The minimal level of significance was chosen as p<0.05.
Results

Three pairs of samples were excluded due to technical reasons. Sections from the fetlock (7 irradiated and 7 controls), the loin (7 irradiated and 7 controls) and the hamstring (4 irradiated and 4 controls) areas, were examined. Macroscopic erythema was observed in the tissue receiving the highest dose (the loin) approximately 5-10 min after irradiation.

Histological examination

The following pathological changes were found: epidermal necrosis with coagulation necrosis of underlying dermis and adnexal destruction, intraepidermal spongiosis and clefts, and subepidermal clefts. The spongiosis and clefts could be present focally or diffusely in the sections. No cell infiltrates were found in dermis or epidermis and dermal blood vessels had a similar appearance in all sections except in the necrotic dermis. Four different categories were created based on the findings. The first category (0) showed no visible changes (Fig.1a). The second (I) was characterised by multifocal, mild spongiosis in the basal epidermis (Fig.1b) with focal subepidermal cleft formations, and the third (II) category presented a diffuse spongiosis in the hair follicle epithelium and of the epidermis with intra- and subepidermal vesicles (Fig.2b) including eosinophilic material. In the most severely affected specimens, an epidermal necrosis with a very thin epidermal layer and coagulation necrosis of underlying dermis was found. This included destruction of hair follicles, sebaceous and sweat glands with necrotic epithelium and necrosis of arrector pili muscles. These specimens were denoted as category III (Fig.3 b,d,f). The relationship between histological lesions and treatment doses is presented in Table 2.

Table 2. Number of horses with morphological changes after irradiation

<table>
<thead>
<tr>
<th>Irradiation Dose (J/cm²)</th>
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<th>Loin (n=7)</th>
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<tbody>
<tr>
<td></td>
<td>Laser</td>
<td>Control</td>
<td>Laser</td>
</tr>
<tr>
<td>Changes</td>
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</tr>
<tr>
<td>0</td>
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<tr>
<td>I</td>
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<td>5</td>
</tr>
<tr>
<td>II</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>III</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

0 = no changes; I = multifocal, mild spongiosis in the basal epidermis with focal subepidermal cleft formations; II = diffuse spongiosis in the hair follicle epithelium and of the epidermis with intra- and subepidermal vesicles including eosinophilic material; III = epidermal necrosis with a thin epidermal layer, and coagulation necrosis of underlying dermis.
Fig. 1. Microscopic sections through the skin of the hamstring area. a) Non-irradiated skin with normal epidermis, dermis and hair follicle, (category 0). b) Multifocal mild spongiosis in basal epidermis (arrows) of skin subjected to defocused CO\textsubscript{2} laser at 91 J/cm\textsuperscript{2}. E = epidermis; D = dermis; F = hair follicle. H&E stained. Bar = 20 μm.

Fig. 2. Microscopic sections through the skin of the fetlock area. a) Non-irradiated skin with normal epidermis, dermis and hair follicle. b) Skin subjected to defocused CO\textsubscript{2} laser at 137 J/cm\textsuperscript{2}. Diffuse spongiosis of epidermis and hair follicle epithelium (arrows). Intraepidermal (blue arrowhead) and subepidermal (arrowhead) vesicles can be seen. E = epidermis; D = dermis; F = hair follicle. H&E stained. Bar = 20 μm.
Fig. 3. Microscopic sections through the skin of the loin area. a, c, e) Non-irradiated skin with normal epidermis, dermis and hair follicle. b, d, f) Skin subjected to defocused CO₂ laser at 450 J/cm². b) Epidermal necrosis with thin epidermis (star), coagulation necrosis of underlying dermis and destruction of arrector pili muscle (blue arrowhead) can be seen. d) Note necrosis with vacuolization of sebaceous glands (arrows) and epithelial necrosis with thinning of hair follicle (arrowheads). f) Destruction of epithelium in hair follicle with clefts (arrowheads) and necrotic eosinophilic sweat glands (blue arrows) are found. E = epidermis; D = dermis; F = hair follicle; S = sebaceous glands; SW = sweat glands. H&E stained. Bar = 20 μm.
**Thickness of epidermis**

A total of 17-102 measurements/section (39±18) were obtained.

Epidermal thickness in the three different locations is shown in Table 3. Non-irradiated skin (controls) presented a significant variation in thickness of epidermis between fetlock (64.2±13.8 μm), loin (30.9±4.2 μm) and hamstring (25.3±2.5 μm) areas. Hence, the areas were evaluated separately. The epidermis was significantly thinner after irradiation in laser-treated loin (450 J/cm²) compared to the non-irradiated loin area (21.8±8.4 vs. 30.9±4.2 μm, respectively, p<0.05). Epidermal thickness was not affected in the other two treatment groups.

Table 3. *Epidermal thickness in different locations (μm)*

<table>
<thead>
<tr>
<th>Dose (J/cm²)</th>
<th>Hamstring (n=4)</th>
<th>Fetlock joint (n=7)</th>
<th>Loin (n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>91</td>
<td>25.3±2.5</td>
<td>64.2±13.8</td>
<td>30.9±4.2</td>
</tr>
<tr>
<td>137</td>
<td>26.8±4.0</td>
<td>68.0±16.5</td>
<td></td>
</tr>
<tr>
<td>450</td>
<td></td>
<td></td>
<td>21.8±8.4*</td>
</tr>
</tbody>
</table>

Results are presented as mean ± standard deviation (sd). * Significantly different from non-irradiated loin.

**Discussion**

Today, different types of laser therapy are widely used in a variety of injuries like tendonitis, traumatic arthritis and wounds (Bromiley, 1991; Ramey & Basford, 2000; Lindholm *et al.*, 2002; Sutton, 2003). However, to our knowledge, no studies have been conducted on the histological effects of defocused CO₂ laser on equine skin. The present study examined the histological changes in equine skin after defocused CO₂ laser irradiation at hamstring, fetlock and loin areas (doses 91, 137 and 450 J/cm², respectively). Mild changes with focal spongiosis and subepidermal clefts were occasionally found in the 91 J/cm² dose and more severe changes with diffuse subepidermal clefts in the 137 and 450 J/cm² doses. Also epidermal and dermal necrosis could be found in many of the samples from the loin area (450 J/cm²). Despite these morphological changes, erythema was detected only after the 450 J/cm² dose.

When designing the study protocol, the doses were chosen in an interval resembling therapeutic (91 J/cm²) and surgical doses (450 J/cm²). However, the latter is also possible to attain in practice, if the irradiation area is accidentally decreased. The 137 J/cm² dose was chosen as being a dose with the same output power and irradiation area as the 91 J/cm², but with a longer exposure time. The fetlock and loin areas are locations where laser treatment is performed, and the hamstring area was chosen as a site where it was possible to investigate underlying skeletal muscle perfusion, as the present study was part of another investigation (Bergh *et al.*, 2006).
We found an exposure time-dependent difference in the amount of morphological lesions between irradiation with 91 J/cm² and 137 J/cm², and a dose-dependent difference between all doses. It has been reported that CO₂ laser irradiation (0.69-13.6 W/cm²) induces exposure time and dose-dependent macroscopic lesions in the porcine skin, similar to thermal burns (Brownell et al., 1969). Similar to the results of the present study, dose-dependent lesions such as shrinking and flattening of epidermal cells (epidermal necrosis), separation between epidermis and dermis, and dermal necrosis was reported by Laor et al. (1969), after irradiation of mice skin (7-100 J, 694 nm and 300-900 J, 1060 nm). Transmission of heat through the hair shaft may be an explanation to the increased damage at the hair bulb level, apparently present in an undamaged dermis (Laor et al., 1969). Furthermore, similar changes are reported in humans (CO₂, 1.6-230 J/cm², Kamat et al., 1986) and after skin ablation (Walsh, 1988). The more severe changes after the 137 J/cm² compared to the 91 J/cm² dose in our study were possibly latent thermal injuries, explained by a cumulative effect of repeated exposures of heat (Moritz & Henrique 1947; Grover et al., 1999), as the only difference in dose was the number of scanning repetitions. However, comparison between studies is often difficult since specification of treatment parameters is often inadequate (Basford, 1995; Basford et al., 2000) and different wavelengths have dissimilar absorption- and penetration patterns (Hecht, 1992).

It is not surprising that the superficial layers of the skin are affected since CO₂ laser light is almost mainly absorbed within the outer 20-40 μm of the tissue (Hecht, 1992). Previously reported thermal damage to skin varies from <0.1 to 0.5 mm (Pearce & Thomsen, 1995; Rizzo et al., 2004) up to 1 mm (Andersson, 1994) and results from heat conduction (Zweig et al., 1990). In vitro incision of equine skin with surgical CO₂ laser (65,799 W/cm²) causes a mean thermal injury zone of 187±152 μm (Palmer & McGill, 1992). To minimize latent thermal injury, pulse durations must be shorter than the thermal relaxation time; whereas longer pulses cause lateral damage due to heat conduction (McKenzie, 1983; Walsh et al., 1988). The main histological changes in the present study were detected in the epidermal basal layer, although the infrared light should be uniformly absorbed by any water-containing cell. The structural differences between epidermis and dermis, as well as the vulnerability of the germinal cells that are not as highly keratinized as those near the surface, may influence the thermal effect (Kamat et al., 1985). It is likely that heat conduction occurred in the 137 J/cm² dose of the fetlock area, since subepidermal clefts were detected below the major absorption depth of 20-40 μm, as the mean thickness of epidermis in the fetlock area was 64.2 μm. Also, the biopsies from the loin area, irradiated with 450 J/cm², showed changes in the underlying dermis below 40 μm from the epidermal surface (epidermis of loin area; 30.9 μm). The present study found a location-dependent difference in the thickness of normal, non-irradiated epidermis (controls), which corresponds to earlier studies on equine epidermis (Taluktar et al., 1972). It is possible, but not likely, that the thickness of epidermis had an influence on the amount of morphological changes as the risk for thermal injury decreases with increasing epidermal and dermal thickness (Geiser & Walker, 1984; Jiang et al., 2002).
However, the main thermal effect of the CO$_2$ laser is most likely connected to its absorption pattern and then conducted to surrounding tissue, in contrast to conventional heating, which is usually more uniformly distributed in tissue (Polla & Andersson, 1987). Furthermore, the more severe changes observed at the 137 J/cm$^2$ dose, compared to the 91 J/cm$^2$, were detected in a thicker epidermis.

It is possible that the changes observed could have become aggravated if longer time than the present 90 min had elapsed between the irradiation and sampling of the biopsies, as the full extent of changes are not detected until 24-72 hours (Hinshaw, 1968; Thomsen, 1991) and are more marked in thermal injury caused by longer exposures at lower irradiance (Henriques, 1947; Hinshaw, 1968). A secondary inflammatory reaction with margination of leukocytes was not seen and not expected in the present study. The lesions of type I and II were concentrated to the epidermis, which lack vessels and type III lesions showing dermal necrosis as a result of thermal injury. This will induce direct damage to endothelial cells as a “delayed prolonged leakage” that begins after 2 to 12 hours (Mitchell & Cotran, 2003).

Our present findings have implications to the understanding of the thermal effects of defocused CO$_2$ laser irradiation on equine skin. It provides data for assessing exposure limits and provides an insight into what could be expected as a result of an accidental exposure at higher levels. The changes in skin morphology seen after irradiation with 91 J/cm$^2$ dose were mild, but with 137 J/cm$^2$ dose, moderate lesions, compatible to thermal injury, were observed. An inflammatory reaction secondary to a thermal injury, which leads to increased blood perfusion, has been reported (Moritz & Henrique, 1947; Geiser & Walker, 1984). The inflammatory reaction may function as a counter-irritant with a possible influence on pain perception (Le Bars, 2002). Our study also showed morphological skin lesions after irradiation with doses of 137 and 450 J/cm$^2$, which correspond to moderate/severe thermal injury and could be classified as hazardous to the skin. This information points out the importance of a careful selection of laser parameters when designing a treatment protocol in equine practice, in order to prevent negative side effects of the defocused CO$_2$ laser therapy.

**Conclusion**

This study revealed minor superficial morphological changes in equine epidermis after irradiation with 91 J/cm$^2$ doses of defocused CO$_2$ laser compared to severe changes with necrosis and a significantly thinner epidermis, when irradiation doses of 450 J/cm$^2$ were applied. Hence, doses higher than 91 J/cm$^2$ on equine skin, delivered at 16 W for 4 min and on 42 cm$^2$ surface area should be restricted. Our future studies will focus on the implementation of present results in clinical settings, thus examining the clinical effect of defocused CO$_2$ laser on pain perception and tissue regeneration.
Acknowledgements

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Manufacturers’ addresses

1Orion Pharma, Finland.
3Pharmacia and Upjohn, Stockholm, Sweden.
4Chassot and Cie AG, Switzerland.
5Abbott, Solna, Sweden.
6Astra, Södertälje, Sweden.
7Apoteksbolaget AB, Umeå, Sweden
8EL.EN. SRL, Firenze, Italy.
9Gamma Optronic AB, Uppsala, Sweden.
10Miltex Instrument Company, INC. Lake Success, New York, USA.
11Leica Microsystems Nussloch GmbH, Heidelberg, Germany
12Bergström Instrument AB, Stockholm, Sweden
13Statsoft Scandinavia AB, Uppsala, Sweden.

References


Defocused CO$_2$ Laser Therapy in Traumatic Arthritis of the Fetlock Joint: A Randomised Clinical Study

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Key words: CO$_2$ laser therapy, traumatic arthritis, horse, rehabilitation, accelerometer technique, substance P, Met-enkephalin-Arg-Phe

Abstract

A prospective, blinded, randomised and controlled study was conducted in horses with traumatic arthritis of the fetlock joint in order to evaluate the efficacy of defocused carbon dioxide (CO$_2$) laser therapy. Sixteen horses were randomly assigned to two groups; active laser (an output power of 16 W, scanned over 42 cm$^2$, an average dose of 91 J/ cm$^2$) and sham laser (the laser beam directed at the non-reflecting floor). All horses received a total of five treatments during one week. Horses were evaluated with respect to the degree of initial lameness by conventional lameness examination and an objective accelerometer technique before onset of treatment, and at 1 and 3 weeks. The concentration of substance P (SP), prostaglandin E$_2$ (PGE$_2$) and Met-enkephalin-Arg-Phe (MEAP) in synovia was also assessed. Our study demonstrates that there was no significant difference in lameness score between the laser and sham treated group before treatment and at 1 and 3 weeks after the initial examination.
Moreover, no significant difference in the concentration of SP, PGE\textsubscript{2} and MEAP in synovia in the active laser group compared to the sham treated group was observed. In conclusion, this study suggests that treatment with defocused CO\textsubscript{2} laser in the management of traumatic arthritis of the fetlock joint is not statistically better than sham treatment at reducing the grade of lameness as evaluated by conventional lameness examination and accelerometer technique.

**Introduction**

Fetlock arthritis has been identified as the most common diagnosis in a large number of Swedish insured riding and leisure horses (Penell *et al.*, 2005). Traumatic arthritis is often accompanied by synovitis, with symptoms of synovia effusion, increased skin temperature over the joint, a palpable thickening of the joint capsule, a decreased joint range of motion and lameness. The inflammation causes distress to the horses and limits their ability to function as competition or leisure horses. It is well known that the concentration of the pro-inflammatory substance P (SP) and prostaglandin E\textsubscript{2} (PGE\textsubscript{2}) are higher in synovia of horses with joint disease compared to normal joints (Gibson *et al.*, 1996; Hardy *et al.*, 1997; Kirker-Head *et al.*, 1999). Capsule, ligaments and synovial membranes are innervated by SP-containing nerve terminals (Caron *et al.*, 1992; Bowker *et al.*, 1993; Nixon & Cummings, 1994) and SP is released after noxious stimuli to primary C-afferents (Duggan *et al.*, 1987; Duggan *et al.*, 1988). Besides causing vasodilatation, SP (Schaible, 2006) and PGE\textsubscript{2} (May *et al.*, 1994) increase the mechanosensitivity in numerous afferents. Hence, pain is evoked by mechanical stimuli such as palpation, movement within normal range of motion and weight loading, which normally would not elicit pain (Schaible, 2006). The opioid Met-enkephalin-Arg-Phe (MEAP) is derived from pro-enkephalin and found in the CNS of rats subjected to induced inflammation. MEAP is suggested to be a mediator of descending antinociceptive pathways (Rosen *et al.*, 2000). An earlier study on horses revealed opioid receptors in the synovial membranes and that the opioids can decrease inflammatory-induced pain through inhibition of release of SP from peptidergic neurons (Sheehy *et al.*, 2001).

Treatment of traumatic arthritis focuses on reduction in inflammation, pain relief, and functional recovery by inhibiting cartilage deformity factors. The treatment of choice is often intra-articular injections with corticosteroids and/or sodium hyaluronate, sometimes complemented with systemic non-steroid anti-inflammatory drugs. During the last decade, non-pharmacological therapies such as physical therapy have received an increasing interest. Current treatments also include complementary methods, out of which laser therapy is only one modality used in the treatment of traumatic arthritis in horses.

Laser therapy comprises a broad field, with multiple laser devices emitting irradiation at different wavelengths and with different output effects. Laser therapy can be divided into surgical lasers (high-power lasers) and lasers used for biomodulation, i.e. low-level lasers.
Devices like the CO₂ laser, originally used in surgery, are today used as biomodulating lasers; with a defocused beam and low output effect. The CO₂ laser emits infrared light, which causes a thermal effect that is not observed in low-level laser therapy. There is divergent information about the clinical effects of laser therapies. Some studies have presented positive results on musculoskeletal injuries in humans (Bertolucci & Grey, 1995; Basford et al., 1999; Bjordal et al., 2001; Bjordal et al., 2003; Gur et al., 2003; Gur et al., 2004; Chow & Barnsley, 2005), while others have not (Basford et al., 2000; Brosseau et al., 2005). It is difficult to establish consensus on laser therapy as many studies are of questionable methodological quality (Beckerman et al., 1992; Basford et al., 1995), and heterogenic in regard to the protocol used, parameters analysed and type of injury treated (Basford, 1995; Bjordal et al., 2003; Chow & Barnsley, 2005). The exact mechanisms of action are not completely understood although a number of mechanisms have been postulated including: improved local circulation (Bergh et al., 2006), anti-inflammatory effects (Campana et al., 2004; Bjordal et al., 2006), enhanced cartilage and bone healing (Chen & Zhou, 1989; Tsai et al., 1997; Cho et al., 2004), stimulating peripheral nerves and analgesic effect (Wesselmann et al., 1991; Baxter et al., 1994). To our knowledge, the only study published on defocused CO₂ laser therapy in horses (Lindholm et al., 2002) reports on improvements of lameness after treatment of traumatic fetlock arthritis (60 J/cm²) superior to the effect of intra-articular injection of bethametasone together with sodium hyaluronate.

Due to the promising observations mentioned above, the general objective of the present investigation was to describe the effects of defocused CO₂ laser on equine traumatic arthritis in a randomised controlled study. The primary objective was to examine the efficacy of defocused CO₂ laser therapy as a rehabilitation tool for pain relief, judged by conventional lameness examination and use of an accelerometer technique. The second objective was to evaluate its pain modulating and anti-inflammatory effect by examining the levels of SP, PGE₂ and MEAP in synovia. It was hypothesised that treatment with defocused CO₂ laser on traumatic arthritis in horses decreases the degree of lameness, as evaluated by conventional lameness examination and accelerometer technique.

**Material and methods**

**Horses**

Of the 45 horses in total presented for the first assessment, 29 did not meet the inclusion criteria, i.e. a total of 16 horses were included in the study. They were all admitted to the Large Animal Clinic, SLU, due to lameness of a forelimb.

The inclusion criteria were that the horse should: 1. be used for riding/trotting, 2. be over 3 years of age, 3. have an initial lameness graded between 0.5-2 degrees in one or both forelimbs, 4. show a ≥75% decrease in lameness after intra-articular
anaesthesia of the fetlock joint and 5. demonstrate only normal to minor findings on radiographs of the actual joint. The following horses were excluded from the study: 1. horses treated for fetlock joint arthritis within the last 3 months, 2. horses with other signs of lameness than fetlock joint arthritis of a forelimb, 3. horses with wounds in the fetlock joint area of the lame limb, 4. pregnant mares, 5. horses with signs of general infection or other illnesses that could interfere with the results, 6. horses on medication, and 7. horses with impaired sensibility of the fetlock joint area. Eight horses received laser treatment and eight served as controls. The basic data about the horses are presented in Table 1.

The Ethical Committee on Animal Experiments in Uppsala, Sweden approved the study, and written informed consent was obtained from each horse owner prior to the study.

Table 1. Baseline characteristics for both laser and sham groups

<table>
<thead>
<tr>
<th>Breed</th>
<th>Age</th>
<th>Sex</th>
<th>Main activity</th>
<th>Duration of lameness</th>
<th>Previous lameness</th>
<th>Initial Flexion grade</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Active laser group</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Icelandic</td>
<td>11</td>
<td>g</td>
<td>training/competition</td>
<td>&gt;71 d</td>
<td>yes</td>
<td>0.5</td>
<td>1.5</td>
</tr>
<tr>
<td>W.b.</td>
<td>7</td>
<td>m</td>
<td>training</td>
<td>0-7 d</td>
<td>yes</td>
<td>0.5</td>
<td>1.5</td>
</tr>
<tr>
<td>Icelandic</td>
<td>13</td>
<td>g</td>
<td>training</td>
<td>&gt;71 d</td>
<td>yes</td>
<td>0.5</td>
<td>3.0</td>
</tr>
<tr>
<td>Connemara</td>
<td>8</td>
<td>g</td>
<td>walks/ training</td>
<td>29-42 d</td>
<td>yes</td>
<td>0.5</td>
<td>2.5</td>
</tr>
<tr>
<td>Stb Tr.</td>
<td>5</td>
<td>g</td>
<td>walks</td>
<td>&gt;71 d</td>
<td>no</td>
<td>0.5</td>
<td>2.5</td>
</tr>
<tr>
<td>Pony cross</td>
<td>9</td>
<td>m</td>
<td>training</td>
<td>8-14 d</td>
<td>no</td>
<td>0.5</td>
<td>3.0</td>
</tr>
<tr>
<td>Russ</td>
<td>10</td>
<td>m</td>
<td>walks</td>
<td>&gt;71 d</td>
<td>-</td>
<td>0.5</td>
<td>3.5</td>
</tr>
<tr>
<td>W.b.</td>
<td>4</td>
<td>g</td>
<td>walks/ training</td>
<td>15-28 d</td>
<td>yes</td>
<td>0.5</td>
<td>2.0</td>
</tr>
<tr>
<td><strong>Median</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>8.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td><strong>Sham laser group</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Icelandic</td>
<td>10</td>
<td>m</td>
<td>training</td>
<td>0-7 d</td>
<td>no</td>
<td>0.5</td>
<td>2.0</td>
</tr>
<tr>
<td>Stb Tr.</td>
<td>6</td>
<td>m</td>
<td>training</td>
<td>43-70 d</td>
<td>yes</td>
<td>0.5</td>
<td>2.0</td>
</tr>
<tr>
<td>Pony cross</td>
<td>10</td>
<td>g</td>
<td>training/competition</td>
<td>&gt;71 d</td>
<td>yes</td>
<td>0.5</td>
<td>2.5</td>
</tr>
<tr>
<td>Pony cross</td>
<td>9</td>
<td>g</td>
<td>training/competition</td>
<td>8-14 d</td>
<td>yes</td>
<td>0.5</td>
<td>3.0</td>
</tr>
<tr>
<td>Pony cross</td>
<td>18</td>
<td>g</td>
<td>training/competition</td>
<td>43-70 d</td>
<td>no</td>
<td>0.5</td>
<td>3.0</td>
</tr>
<tr>
<td>W.b.</td>
<td>8</td>
<td>g</td>
<td>training</td>
<td>0-7 d</td>
<td>no</td>
<td>0.5</td>
<td>2.5</td>
</tr>
<tr>
<td>Stb Tr.</td>
<td>6</td>
<td>g</td>
<td>walks/training</td>
<td>15-28 d</td>
<td>-</td>
<td>0.5</td>
<td>1.0</td>
</tr>
<tr>
<td>Lipizzaner</td>
<td>11</td>
<td>g</td>
<td>training/competition</td>
<td>8-14 d</td>
<td>no</td>
<td>1.5</td>
<td>2.5</td>
</tr>
<tr>
<td><strong>Median</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>9.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

Stb Tr. = standardbred trotter; W.b. = warmblooded riding horse; g = gelding; m = mare; d = days; lameness score 0-5 (0 = no lameness, 5 = non weight-bearing); - = not registered. Data is presented as median and range.
Techniques

Laser

A defocused CO₂ laser (10600 nm, KSV 25S; EL.EN. SRL, Firenze, Italy) was used in the study (Table 2). As guiding light, a HeNe source (633 nm) was emitted continuously at 1.2 mW. The same laser was used for the sham treatment. The laser device was calibrated regularly by an independent technician, and an external detector (LaserMate Detector, COA-33-0191-000; Gamma Optronic AB, Uppsala, Sweden) was used to measure the intensity of the laser beams before and after each treatment.

Table 2. Lasing medium parameters (KSV 25S Laser device)

<table>
<thead>
<tr>
<th></th>
<th>CO₂</th>
<th>HeNe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wavelength</td>
<td>10.6 µm</td>
<td>632.8 nm</td>
</tr>
<tr>
<td>Output power</td>
<td>16 W</td>
<td>1.2 mW</td>
</tr>
<tr>
<td>Mode of application</td>
<td>scanning</td>
<td>scanning</td>
</tr>
<tr>
<td>Diameter of beam at source</td>
<td>6 mm</td>
<td>0.61 mm</td>
</tr>
<tr>
<td>Divergence</td>
<td>1.5 mrad</td>
<td>2.0 mrad</td>
</tr>
<tr>
<td>Distance to irradiated area</td>
<td>1 m</td>
<td>1 m</td>
</tr>
<tr>
<td>Irradiated area</td>
<td>42 cm²</td>
<td>42 cm²</td>
</tr>
<tr>
<td>Site of application</td>
<td>fetlock joint</td>
<td>fetlock joint</td>
</tr>
<tr>
<td>Treatment time</td>
<td>4 min</td>
<td>4 min</td>
</tr>
<tr>
<td>Average dose</td>
<td>91 J/cm²</td>
<td>0.007 J/cm²</td>
</tr>
</tbody>
</table>

Accelerometers

Two two-axis differential-capacitance accelerometers (ADXL250, Analog Devices, USA) were mounted perpendicular to each other on a board together with analogue components, memory, a/d-converters, battery and RF-equipment, which altogether where gathered in a box. These transducers were fixed to pockets in boots, girth and the neck piece (Fig. 1).
Fig. 1. Accelerometer devices adapted to the neck, withers, croup and legs.

Transducers were applied to the four legs, withers, neck and croup, with approximately horizontal and vertical measuring axes, and an offset set to zero. The accelerometers measured acceleration, ± 50 g, with a resolution of 0.01 g and a sampling frequency of 1000 Hz in three directions; x, y and z, thus creating three-dimensional acceleration data. The accelerometers measure the instant change of velocity during a given interval that corresponds to the acceleration applied at the position of the accelerometer. The acquisition duration was 10 s. Data were sampled synchronously at all seven transducers with software developed by Rolab AB (Glunten, Uppsala, Sweden). Data sampling was triggered telemetrically and after the recordings a number of data registrations were downloaded from the transducers devices to a PC via serial interface for further analysis.

Accelerometer data analysis
Data from the dorsoventral and longitudinal axis of the transducer on the withers and the dorsoventral axis of the transducer on the neck were selected for further analysis. Power spectrum for the three-time series and subsequently the quotient between the first and second harmonic was calculated. Data from each registration of the initial lameness were averaged.

The second harmonic (at approximately 2.5 Hz) represents the step frequency, which in trot is half of the stride frequency. If the trot is symmetrical this will be the dominant harmonic. In case the horse is lame, i.e. asymmetrical, a harmonic (the first harmonic) equal to the stride frequency will start to increase. Consequently, the quotient between the first and second harmonic will constitute an objective measure of the gait asymmetry in trot. The three quotients were summed and used as an objective measurement of the gait asymmetry.
Procedures

Study design

Recruitment of horses took place from September 2001 to May 2005. Sixteen horses were randomly assigned to the two groups by drawing envelopes labelled “A” (Group 1: active laser; 8 horses) and “B” (Group 2: sham laser; 8 horses), with a randomly permuted block size of four, to prevent unequal distribution by chance for seasonal factors.

The horse owners were informed about the project in advance. The horses were examined according to a conventional lameness protocol by an experienced clinician, blinded to the treatment. The horses were observed at the walk and trot on a straight line, and lameness was assessed on a 0-5 scale (Åsheim & Lindblad, 1976). Thereafter, accelerometer devices were fixed to the horse and a flexion test of the phalanges of the lame leg was performed followed by a new lameness recording including accelerometer registrations. The lameness examination was also recorded with a digital video camera. Afterwards, a blood sample was drawn from the jugular vein.

In horses with bilateral forelimb lameness, the most symptomatic forelimb was selected for treatment. After the “boot” on the lame leg had been removed, the lateral aspect of the fetlock joint was surgically prepared, a synovia sample was collected and intra-articular analgesia (7 ml mepivacain 20mg/ml, and 21 gauge needle) was performed. The effect of the local analgesia was evaluated after 15 min, after which new accelerometer registrations were carried out before and after a second flexion test. If the analgesia blocked the initial lameness by ≥ 75%, radiology of the fetlock joint was carried out to rule out any moderate or major defects. Thereafter, the horse owner signed an agreement confirming their horse to participate in the study.

The horses were stabled at the clinic for one week and were walked by hand for 2 x 10 min daily. During this week, all horses received a standardised treatment regimen that consisted of five laser or sham treatments, standing in stocks. None of the horses were sedated on any occasion. The laser system was set to give continuous output power of 16 W, scanned over 42 cm², with an average dose of 91 J/cm². Three, lateral, dorsal and medial, aspects of the fetlock joint were irradiated for 4 min, respectively. The control (sham) group followed the same procedure as the laser-treated group, except that the laser beam was directed at the non-reflecting floor. All horses but one were examined by the same clinician and all horses were treated by the same therapist.

Follow-up procedure

The horses were re-examined with the initial protocol and by the same veterinarian, 7 and 21 days after the initial examination and the study was completed after 3 weeks. The degree of lameness was considered as the most important outcome measure and was assessed by conventional lameness evaluation and with an objective accelerometer technique.
The results observed during conventional lameness examination of the initial lameness were classified by means of a four-grade semiquantitative scale: fully improved (no initial lameness and flexion test <1.0°), partially improved (initial lameness improved ≥50%), not improved (same initial lameness), and worse (an increase in initial lameness). The results observed with accelerometer technique were classified by means of a two-grade semiquantitative scale: improved (the quota decreased) or not improved (the quota increased). Second outcome measures were blood and synovia analyses; analysed for leukocytes and protein, as well as levels of MEAP, PGE₂ and SP analysed in synovia by radioimmunoassay (RIA) and enzyme immunoassay (EIA).

The horses that improved significantly after the week of treatment were sent home on a two week daily convalescence program of box rest completed with 2 x 20 min walk by hand. The horses that did not show improvement at the day 7 examination, were offered a complementary treatment; normally with intra-articular injection of sodium hyaluronate and corticosteroids. These horses were considered excluded from the future follow-up examination, but were re-evaluated after 21-27 days after injection. The clinician was unaware of the code for laser or sham treatment until the data analysis for all horses was completed.

**Blood and synovia samples**

Blood samples were collected from the jugular vein and synovia samples were collected after surgical preparation from the lateral aspect of the fetlock joint. Vials containing EDTA were used for measurement of white blood cell count and serum vials were used for determination of total plasma protein concentration. The blood and synovia analytes were assayed according to the routine methods used at the Division of Clinical Chemistry, SLU. Additional synovia samples were centrifuged 2 x 20 min (600 x g). The supernatant was removed, frozen and stored at -80°C, until analysis.

**Measurements of SP and MEAP**

The synovial fluid samples (230 µl) were acidified by adding 200 µl of 0.1 M HCl and homogenised by ultrasonification. The homogenates were diluted (1:10) with 0.1 M of formic acid /0.018 M pyridine, pH 3.0 (buffer I) and subsequently centrifuged for 2 minutes at 3000 x g (4°C). The supernatant fractions were purified by ion-exchange chromatography. Small plastic columns were packed with SP-Sephadex C-25 gel (GE Healthcare, Uppsala, Sweden, packed gel volume = 1 ml) and washed with 20 ml buffer I before the samples were added. After additional washing with 10 ml of buffer I and 5 ml of 0.1 M formic acid /0.01 M pyridine (buffer II), the fraction containing SP and MEAP was eluted with 4 ml of 1.6 M formic acid /1.6 M pyridine, pH 4.4 (buffer V). All buffers (I, II, V) contained 0.01% mercaptoethanol. The eluates were evaporated in a Speed Vac centrifuge (Savant, Hicksville, NY, U.S.A.) and subsequently analysed by radioimmunoassay (RIA).

The radioimmunoassay for SP and MEAP were based on the charcoal adsorption technique and were conducted as described (Hallberg et al., 2000; Johansson et al., 2000).
For all RIAs the antibodies were raised in rabbits against the peptide-thyroglobulin conjugate and the $^{125}$I-labelled Met-enkephalin-Arg$^6$-Phe$^7$ or Tyr$^8$-substance P were used as tracers. Details (including cross-reactivity and detection limits) for the substance P RIA was performed according to Nyberg and co-workers (Sharma et al., 1990; Hallberg et al., 2000) and for analysis of MEAP was performed according to Johansson and co-workers (Johansson et al., 2000). The intra-assay variance was 8.5% for SP and 40% for MEAP.

Measurements of PGE$_2$
The concentration of PGE$_2$ was determined by direct enzyme immunoassay (EIA) as previously described by Skarzynski et al. (2000). Cross-reactivity for the antiserum was: PGE$_2$ 100%; PGE$_1$ 18%; PGA$_1$ 10%; PGA$_2$ 4.6%; PGB$_2$ 6.7%; PGD$_2$ 0.13%; PGE$_2$-$\alpha$ 2.8%; PGJ$_2$ 14% and 15-keto PGE$_2$ 0.05%. The PGE$_2$ standard curve ranged from 0.08ng/ml to 20 ng/ml. The intra-assay coefficient of variation was 8.4%.

Statistical analysis
Statistica 6.0 (Statsoft, 2001; Statsoft Scandinavia AB, Uppsala, Sweden) was used for data analysis, and results are presented as medians and range. Statistical calculations were performed with non-parametric Wilcoxon within groups and Mann-Whitney U tests between groups. Statistical significance was accepted at $p<0.05$.

Results

The most common reason for exclusion was lameness originating from other joints and surrounding tissues, thereby interfering with the outcome assessment. The median age of active laser group was 8.5 (4-13) years, and sham group 9.5 (6-18) years. There was no major difference in any baseline characteristics of the groups (Table 1). No treatment sessions were missed in any horse, and none of the 16 horses was withdrawn from the study. No complications or side effects were observed.

The results of the blood and synovia analyses are presented in Table 3. The measurement of white blood cell count and protein in blood did not show significant differences between the laser and sham treated group either before or after treatment. There was no significant difference in the concentration of SP, PGE$_2$ and MEAP in synovia between the laser group and the sham group, however, in 5 out of 7 horses in the laser-treated group the concentration of MEAP increased, compared to 2 out of 5 horses in the sham-treated group.
Table 3. Results from blood and synovia analyses in horses with traumatic arthritis of the fetlock joint irradiated with laser or sham laser (Laser group = 91 J/cm², sham group = 0 J/cm²)

<table>
<thead>
<tr>
<th></th>
<th>Laser, n=7</th>
<th></th>
<th>Sham, n=8</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before</td>
<td>After</td>
<td>Before</td>
<td>After</td>
</tr>
<tr>
<td><strong>Blood</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B-LPK, 10x9/L</td>
<td>7</td>
<td>7</td>
<td>8</td>
<td>6</td>
</tr>
<tr>
<td>S-Protein, g/L</td>
<td>60</td>
<td>62</td>
<td>60</td>
<td>62</td>
</tr>
<tr>
<td><strong>Synovia</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leuk, 10x6/L</td>
<td>142</td>
<td>256</td>
<td>278</td>
<td>322</td>
</tr>
<tr>
<td>Protein, g/L</td>
<td>8.5</td>
<td>5</td>
<td>8.5</td>
<td>5</td>
</tr>
<tr>
<td>Substance P, fmol/ml</td>
<td>25</td>
<td>23</td>
<td>25</td>
<td>19</td>
</tr>
<tr>
<td>MEAP, fmol/ml</td>
<td>44</td>
<td>67</td>
<td>72</td>
<td>47</td>
</tr>
<tr>
<td>PGE2, pg/ml</td>
<td>1489</td>
<td>1490</td>
<td>3113</td>
<td>1654</td>
</tr>
</tbody>
</table>

B-LPK = Blood leukocytes; S-Protein = Serum protein; D-Leuk = Synovia leukocytes; Protein = Protein in synovia; MEAP = Met-enkephalin-Arg-Phe; PGE2 = prostaglandin E2.

Data is presented as median and range.

The results from the lameness examination before treatment, after 1 week of treatment and after 3 weeks are summarised in Table 4. There was a reduction in initial lameness score in both groups, but no statistical difference in lameness score between the groups examined by either conventional lameness examination or accelerometer technique was found. The results from complementary treatment are shown in Table 4.
Table 4. Comparison of active laser and sham laser groups in respect to clinical outcomes at baseline, after therapy at weeks 1 and 3 (Laser group: 91 J/cm², sham group: 0 J/cm²)

<table>
<thead>
<tr>
<th>Breed</th>
<th>Baseline Lameness score</th>
<th>Week 1 Lameness score</th>
<th>Lameness grading</th>
<th>Complement treatment</th>
<th>Week 3 Lameness score</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>initial flexion</td>
<td>initial flexion</td>
<td></td>
<td></td>
<td>initial flexion</td>
</tr>
<tr>
<td><strong>Active laser group</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Icelandic</td>
<td>0.5</td>
<td>1.5</td>
<td>n</td>
<td>hyl/cort⁰</td>
<td>e</td>
</tr>
<tr>
<td>W.b.</td>
<td>0.5</td>
<td>1.5</td>
<td>n</td>
<td>hyl/cort¹</td>
<td>e</td>
</tr>
<tr>
<td>Icelandic</td>
<td>0.5</td>
<td>3.0</td>
<td>f</td>
<td>no</td>
<td>0.0</td>
</tr>
<tr>
<td>Connemara</td>
<td>0.5</td>
<td>2.5</td>
<td>n</td>
<td>hyl/cort²</td>
<td>e</td>
</tr>
<tr>
<td>Stb Tr.</td>
<td>0.5</td>
<td>2.5</td>
<td>f</td>
<td>no</td>
<td>0.5</td>
</tr>
<tr>
<td>Pony cross</td>
<td>0.5</td>
<td>3.0</td>
<td>f</td>
<td>no</td>
<td>0.0</td>
</tr>
<tr>
<td>Ru ss</td>
<td>0.5</td>
<td>3.5</td>
<td>p</td>
<td>no</td>
<td>0.0</td>
</tr>
<tr>
<td>W.b.</td>
<td>0.5</td>
<td>2.0</td>
<td>f</td>
<td>none²</td>
<td>e</td>
</tr>
<tr>
<td><strong>Median</strong></td>
<td>0.5 (0.5)</td>
<td>2.5 (1.5-3.5)</td>
<td>0.25 (0-0.5)</td>
<td>0.75 (0.5-2)</td>
<td></td>
</tr>
<tr>
<td><strong>Sham laser group</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stb Tr.</td>
<td>0.5</td>
<td>2.0</td>
<td>f</td>
<td>no</td>
<td>0.5</td>
</tr>
<tr>
<td>Pony cross</td>
<td>0.5</td>
<td>2.5</td>
<td>p</td>
<td>no</td>
<td>0.0</td>
</tr>
<tr>
<td>Pony cross</td>
<td>0.5</td>
<td>3.0</td>
<td>n</td>
<td>hyl/cort¹</td>
<td>e</td>
</tr>
<tr>
<td>Pony cross</td>
<td>0.5</td>
<td>3.0</td>
<td>n</td>
<td>hyl/cort¹</td>
<td>e</td>
</tr>
<tr>
<td>W.b.</td>
<td>0.5</td>
<td>2.5</td>
<td>f</td>
<td>no</td>
<td>0.0</td>
</tr>
<tr>
<td>Stb Tr.</td>
<td>0.5</td>
<td>1.0</td>
<td>n</td>
<td>hyl/cort¹</td>
<td>e</td>
</tr>
<tr>
<td>Lipizzaner</td>
<td>1.5</td>
<td>2.5</td>
<td>p</td>
<td>hyl/cort²</td>
<td>e</td>
</tr>
<tr>
<td>Icelandic</td>
<td>0.5</td>
<td>2.0</td>
<td>f</td>
<td>no</td>
<td>0.0</td>
</tr>
<tr>
<td><strong>Median</strong></td>
<td>0.5 (0.5-1.5)</td>
<td>2.5 (1-3)</td>
<td>0.25 (0-0.5)</td>
<td>0.75 (0-2.5)</td>
<td>0 (0-0.5) 0.75 (0-2)</td>
</tr>
</tbody>
</table>

Stb Tr. = standardbred trotter; W.b. = warmblooded riding horse; Lameness score 0-5 (0 = no lameness, 5 = non weight-bearing); Lameness grading f = fully improved; p = partially improved; n = not improved; w = worse compared to the beginning of the study; hyl/cort = hyaluronic acid and corticosteroids; ^{1} = fully improved; ^{2} = not improved at 21-27 days after injection; e = excluded from the study. Data is presented as median and range.
Discussion

Traumatic arthritis of the fetlock joint was selected as the condition to treat, since it is the most common diagnosis in a material of Swedish insured riding and leisure horses (Penell et al., 2005). The ability of primary synovitis to produce early degradation of articular cartilage in the horse has been described by McIlwraith & van Sickle (1981). Thus, it is a condition with both an inflammatory and pain aspect, for which laser therapy has been proposed to be effective.

In the present study, the application of defocused CO$_2$ laser did not show a more positive clinical outcome compared to the control. The results on clinical efficacy of laser therapy in humans are contradictory. Some studies have shown pain relief (Nivbrant & Friberg, 1992; Bertolucci & Grey, 1995; Basford et al., 1999; Bjordal et al., 2003; Gur et al., 2003), while other studies are more reluctant to the treatment of osteoarthritis (Basford, 2000; Brosseau et al., 2004; Tascioglu et al., 2004; Brosseau et al., 2005).

The present study was controlled, blinded and randomised. The inclusion criteria were chosen to select a homogenous group, which is demonstrated by the similarity of the baseline characteristics. However, due to a limited group of horses, a type II error may have influenced the inconclusive results, although a recovery rate of approximately 80% reported by Lindholm et al. (2002) should have been detected in our material. The evaluation period in our study was relatively short as the participating horses were privately owned.

The dose used in the present protocol was somewhat higher and the treatment period longer than that used by Lindholm et al. (2002). Three sides of the fetlock joint were irradiated, as the area of irradiation can be of importance. However, there is no conclusive evidence that dose-dependency exists, although lower doses of low-level laser therapy have been shown to be as effective as higher doses for reducing pain and improving knee range of motion in humans with osteoarthritis (Gur et al., 2003).

The results in Table 4 show that the rate of improvement is approximately 50%, assessed both subjectively and objectively, and in both the control and treatment group. These results can be compared to other clinical studies on traumatic or induced arthritis, with an improvement or return to health at week 3 that varies between 12 and 80% for intra-articular injection of 0.9% NaCl (Gaustad et al., 1999), sodium hyaluronate (Verschooten & Desmet, 1997), polysulphated glucosaminoglycan & sodium hyaluronate (Gaustad & Larsen, 1995) corticosteroids (Rydell et al., 1970; Vernimb et al., 1977), sodium hyaluronate and corticosteroids together (Rydell et al., 1970), and rest alone (Gaustad et al., 1999).

The diagnosis in the present study was made by examination of initial lameness, flexion tests, intra-articular analgesia and radiographs. The flexion test was primarily used as a diagnostic tool and not in the evaluation of the degree of lameness, since previous studies question the reliability of the test (Ramey, 1997; Verchooten & Verbeeck., 1997; Busschers & van Weeren, 2001). Different ways to evaluate and variations in the horse material examined could be the explanation of the discrepancy of results in previous studies.

As expected, we did not find any significant changes in the levels of total white blood cells (WBC) and protein in blood and synovia before treatment. The levels
of WBC and protein were, however, used to exclude cases with septic background. The horses were not sedated during sampling, thus haemorrhage in the synovia could occur and may have influenced the WBC and total protein analysis.

In animal studies on musculoskeletal injuries, the reduction of pain is manifested as a reduction in lameness. The pain in traumatic arthritis may include multiple components with synovitis as a major origin. Therefore, pain and inflammatory parameters are two appropriate outcome parameters, and the conventional lameness examination in the present study was complemented with two objective methods: accelerometer technique and analyses of the concentrations of SP, PGE\(_2\) and MEAP in synovia. The same clinician, blinded to the grouping of horses, examined 15 of the 16 horses participating in the study, and evaluated the grade of lameness from video recordings for the sixteenth horse, to assure that all horses were evaluated by the same protocol.

Laser therapy is suggested to have an anti-inflammatory effect (Amano et al., 1994; Ulugöl et al., 1997) and Bjordal et al. (2006) reports a reduction in PGE\(_2\) levels after infrared laser therapy of Achilles tendonitis. *In vitro* studies show a similar decrease in the level of PGE\(_2\) after irradiation with visible light (Barberis et al., 1996). This was not observed in the present study, possible because the severity of the joint disease and the grade of lameness were relatively low in the examined horses.

Despite of the fact that there was no statistical difference in the improvement rate between horses receiving laser or sham treatment, a tendency towards an increase in the concentration of MEAP in synovia in the laser group (an increase in 5 out of 7 horses) compared to the sham group (an increase in 2 out of 5 horses) was observed. Although the intra-assay variance was relatively high, the analyses were performed the same day, in the same RIA and with the samples randomised.

Pain relief was correlated to the increase in plasma met-enkephalin induced in humans after acupuncture treatment (Kiser et al., 1983) and increased levels of met-enkephalin in cerebrospinal fluid of rats are correlated to decreased pain scores (Pappas, 1999). SP produces an analgesic effect in rats by inducing a release of met-enkephalin at supra-spinal levels involved in pain control (Naranjo et al., 1982). Other studies have not confirmed any effect on neuropathic pain or concentration of dynorphin after irradiation with IR diode lasers (Parris et al., 1994). However, an increase in MEAP may indicate a pain-relieving effect.

In conclusion, this study suggests that treatment with defocused CO\(_2\) laser in the management of traumatic arthritis of the fetlock joint is not statistically better than sham treatment at reducing the grade of lameness as evaluated by conventional lameness examination and accelerometer technique.

There was no significant difference in the concentration of SP, PGE\(_2\) and MEAP in synovia between the laser group and the sham treated group. Further studies need to be conducted to refine the therapeutic application.
Acknowledgement:

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References


