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A Novel Animal Model to Evaluate Oxygen Derived Free Radical Damage in Soft Tissue

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We present a novel animal model which allows the continuous intra-arterial infusion in one hindlimb of non-anaesthetized rats, without inducing ischemia. Using this model the effect of continuous infusion (1ml/h) for 24 h with tert-butylhydroperoxide (tert-BuOOH) at a concentration of 25mM on soft tissue of the left hind limb was studied and compared to the effect of saline infusion (control group). The tert-BuOOH-infused foot showed increased skin temperature, increased circumference, redness of the plantar skin, impaired function and increased pain sensation, while in the contralateral foot and in rats only perfused with saline these signs of inflammation were absent ($p < 0.01$). Histological analysis of the left gastrocnemius muscle showed edema, muscle cell degeneration with a patchy distribution pattern and vascular damage. All these features increased in severity from 4 to 24 h of tert-BuOOH infusion. After 24 h of tert-BuOOH infusion infiltration of neutrophils in the interstitium was observed. Vascular permeability, expressed as left to right gastrocnemius muscle ^{99m}Tc-IgG uptake ratio, was similarly increased after 4 h (2.09 ± 0.26) and 12 h (2.04 ± 0.08) of tert-BuOOH infusion compared to saline (1.05 ± 0.08) ($p < 0.001$), and further increased after 24 h (3.84 ± 0.13): ($p < 0.001$). In this animal model free radical-related soft tissue damage was induced, by continuous infusion of tert-BuOOH, followed by increasing necrosis and vascular permeability in skeletal muscle coinciding with neutrophilic infiltration.

Keywords: Free radicals, skeletal muscle, tert-butylhydroperoxide, vascular permeability, radionuclide imaging, leukocytes, inflammation, necrosis

INTRODUCTION

Free radicals play an important role in a variety of physiologic and pathophysiologic processes such as: inflammation, transplant rejection, ischemia/reperfusion injury, pulmonary diseases and neurodegenerative diseases.[1–3] Soft tissue damage which may be attributed to free radicals has mainly been analyzed in ischemia/reperfusion animal models and in isolated perfused organs.[4,5] However, next to free radicals,[1,6,7] other inflammatory components[8–10] and energy depletion[11,12] contribute to skeletal muscle necrosis and increased vascular permeability observed in the ischemia/reperfusion models. The fact that these three major factors are closely linked in the pathogenesis of ischemia/reperfusion injury, precludes the assessment of

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the specific role of free radicals on the pathogenesis of soft tissue damage.

In order to evaluate free radical-induced tissue damage more directly, organs have been perfused or injected by radical-generating solutions. Hepatocytes, renal tubules and brain were exposed to tert-butylhydroperoxide (tert-BuOOH), which induced oxidative injury.[13–15] Cellular damage induced by tert-BuOOH consists of lipid peroxidation and mitochondrial dysfunction resulting in cell death.[16] In the cell, tert-BuOOH is metabolized by glutathione (GSH) peroxidase to glutathione disulfide (GSSH) and tert-butanol (tert-BuOH). However, this reducing capacity can be overwhelmed by using an excess tert-BuOOH, leading to the formation of radicals such as tert-butoxyl and tert-butylperoxyl.[17,18]

The aim of this study was to evaluate the direct effect of tert-BuOOH infusion on soft tissue and vascular permeability in one hindlimb of rats. For this purpose, in contrast to the usual local injection or perfusion of isolated organs, we employed continuous intra-arterial infusion in one hindlimb without inducing ischemia in non-anaesthetized animals. During the infusion period, the classical signs of inflammation (rubor, calor, dolor, tumor, and impaired function) were observed.

MATERIALS AND METHODS

Animal Model

Adult male Wistar rats (weight 320–400 g) were anaesthetized with subcutaneously injected atropine (0.25 mg/kg), intramuscular injection of fluanison (10 mg/ml)/fentanyl (0.2 mg/ml) mixture (0.445 mg/kg) and intraperitoneal administration of midazolam hydrochloride (4.5 mg/kg). Rats were anticoagulated intravenously with 100 U/kg heparin sodium. During the surgical procedure, body temperature was maintained between 37°C and 38°C using a heated mat. A skin incision was made in the left thigh. Microsurgically, the superficial epigastric artery and vene were isolated. A polythene cannula (ID 0.28 mm; OD 0.61 mm; Laboratoire Portex, France) was placed retrogradely in the superficial epigastric artery, with the lying tip at the orifice of the femoral artery. By this technique the main arterial circulation of the operated hindlimb remained undamaged. Before operation, the tip of the cannula was thinned to a diameter of 0.4 mm. The cannula was fixed in the artery with braided, uncoated, polyester sutures 6.0 (Dagrofil, Braun, Melsungen, Germany). The insertion site of the cannula in the artery was seeled with glue (Histoacryl, Braun, Melsungen, Germany) to avoid leakage of the infusion fluid. The other end of the cannula was extended subcutaneously over the back of the rat to the head and connected to a stainless steel tube (0.5 × 20 mm). This assembly was then attached to the skull with three 3 mm long M1 stainless steel screws and Carboxylate cement (Durelon, ESPE Dental-Medizin, Seefeld, Germany), allowing the free end of the stainless tube to protrude 0.6 cm from the surface of the cement.[19] To this stainless tube a flexible polythene tubing system was connected by a swivel to an infusor pump. The skin was closed with clips. After the operation, anaesthesia was terminated with an intramuscular injection of naloxone (2 μg/kg).

Result of the surgical procedure was a non-anaesthetized rat connected to a flexible infusion system, through which it was possible to selectively infuse in the arterial circulation of one hindlimb without concurrent ischemia. Infusion (1 ml/h) was started immediately after cannulation of the superficial epigastric artery. Maximal duration of infusion was 24 h. The rats were infused with the free radical donor tert-BuOOH (Sigma, St. Louis, USA) dissolved in saline to a final concentration of 25 mM with heparin (2.5 U/ml) or with saline plus heparin (control group). The experimental protocol was approved by the Animal Ethics Review Board of the Faculty of Medicine, University of Nijmegen.

Analysis of Signs and Symptoms

Skin temperature and circumference of the feet of both hindlimbs were measured, and skin colour of the feet was observed in the tert-BuOOH (n = 15) and saline (n = 10) groups just before the operation and 1, 2, 4, 20 and 24 h after starting the infusion. A mark was placed on both hind paws, to have fixed measurement points.

Skin temperature was measured on the plantar region of both hind paws using a surface electrode (d = 0.6 cm; Keithly, Geneva, Ohio, USA), and the temperature difference between both feet was calculated.

Circumference of the paw was measured at the marked foot, using a string. The percentage increase of the length of the string during the infusion period served as a parameter for contour alterations. Colour of the left plantar foot was observed and compared to the right feet during the experiment.

After 24 of infusion, the function of the left hindlimb was noted as impaired when the rat showed a shuffling gait.

Observation of pain signs was performed preoperatively and after 24 h of infusion in 11 tert-BuOOH infused rats and 6 control rats. Assessment of pain was performed according to the different pain methods based on the classical neuropathic pain model in the rat as described by Bennet et al.[20] Therefore, three different pain forms were observed: spontaneous pain, mechanically induced pain and thermally induced pain.

Spontaneous behaviour of rats was observed in a perspex cage of 25 x 25 x 40 cm after 5 min habituation. During 5 min, the time of different positions of the lesioned paw was noted, according to the scale of Attal et al:[21] 0 = the operated paw is pressed normally on the floor; 1 = the paw rests lightly on the floor and the toes are in a ventroflexed position; 2 = only the internal edge of the paw is pressed on the floor; 3 = only the heel is pressed on the floor and the hind paw is an inverted position; 4 = the whole paw is elevated; 5 = the animal licks the operated paw. The score over the 5 min period provides an index of spontaneous pain intensity for each rat. In the tert-BuOOH and control groups the spontaneous pain was calculated QS t1 + 2 t2 + 3 t3 + 4 t4 + 5 t5/300 s where t1, t2, t3, t4 and t5 are the times (in s) spent in categories 1, 2, 3, 4 or 5, respectively.

In order to quantify mechanical sensitivity of the plantar hind paw, foot withdrawal in response to mechanical stimuli was measured pre-operatively and after 24 h of infusion. Mechanical stimuli were applied with Von Frey filaments (North Coast Medical, San José, U.S.A.) of two different bending forces (5.16 g and 12.5 g). A rat was placed in a perspex cage with a wire mesh floor (26 x 26 x 26 cm). After 5 min accommodation, a Von Frey filament was applied to 10 times (once every 5 s) to the plantar surface of the left foot[22] and the frequency of foot withdrawal was noted. For the measurement of thermal pain: a rat was placed in a perspex cage (31 x 31 x 50 cm) with a heated floor of 40°C[22]. After a behavioural accommodation period of 5 min the pain score as used for spontaneous pain evaluation was calculated over 5 min (see above). Following the heat experiment, the floor was chilled to 4°C and the rat was allowed to accommodate for 5 min. Behaviour of the left hind paw was again observed and scored during 5 min with the spontaneous pain score of Attal et al[21].

Histology

Rats were re-anaesthetized after 4 h (n = 2), 12 h (n = 2) or 24 h (n = 6) of tert-BuOOH infusion or 24 h (n = 6) of saline infusion. Left and right gastrocnemius muscles were dissected and immediately fixed in toto by immersion in cold phosphate buffered paraformaldehyde 4%, pH 7.3, whereafter the animals were killed. From proximal to distal, 4 transversal slices were taken, dehydrated and embedded in Paraplast. Sections of 5 µm in thickness were stained with hematoxylin and eosin (HE). In these sections structural changes in skeletal muscle fibers, blood vessels and surrounding tissues were examined light microscopically.

^{99m}Technetium-IgG Distribution

For analysis of permeability changes of the blood vessels ^{99m}Technetium labelled to hydrazinonicotinamide derivatized polyclonal human immunoglobin G (^{99m}Tc—IgG) (Baxter/Hyland Gammagard, Lessines, Belgium) was used.[24] This radiopharmaceutical was chosen in vue of the higher uptake in inflammatory foci and high in vivo stability of the preparation compared to other available agents.[25,26] The ^{99m}Tc—IgG (7.5 Mbq) was injected through the tail vein 2 h before an experiment was terminated.

Scintigraphy was performed on two rats infused for 24 h with tert-BuOOH. After reanaesthesia, the rats were placed prone on a single—head gamma camera equipped with a parallel-hole, low energy collimator and imaged at 5 min and 2 h post injection. The study at 5 min reflects changes in vascular pooling, while the 2 h images represent extravasation of the radiopharmaceutical.[27]

For establishing the biodistribution, rats were re-anesthaetized after 4 h (n = 5), 12 h (n = 5) or 24 h (n = 5) of tert-BuOOH infusion or 24 h (n = 3) of saline infusion. Left and right gastrocnemius muscle were dissected for measurement of the activity in a shielded well—type gamma counter, after which the animals were killed. To correct for radioactive decay and permit calculation of the uptake of the radiopharmaceuticals in the skeletal muscle as a fraction of the injected dose, aliquots of the respective doses were counted simultaneously. To correct for variances in background clearance, uptake of ^{99m}Tc—IgG was expressed as the left (affected) to right (normal) gastrocnemius muscle uptake ratios.

Statistical Analysis

All quantitative data were expressed as mean ± standard error of the mean. Measurements at the various time points were compared by a repeated measures analysis of variance, followed by a Welch two-tailed t-test for comparison of the tert-BuOOH infused rats with the saline infused rats. Pain scores of the two groups were compared by relating the difference between the post- and pre-operative values of the various groups. The level of significance was set at $p < 0.05$.

RESULTS

Signs and Symptoms

Skin temperature difference between the plantar side of the feet was –0.03 ± 0.03°C before the operation, increasing to 1.10 ± 0.19°C after 1 h tert-BuOOH infusion ($p < 0.001$) (fig. 1A). During 24 h tert-BuOOH infusion, skin temperature remained higher compared to the pre-operative situation,

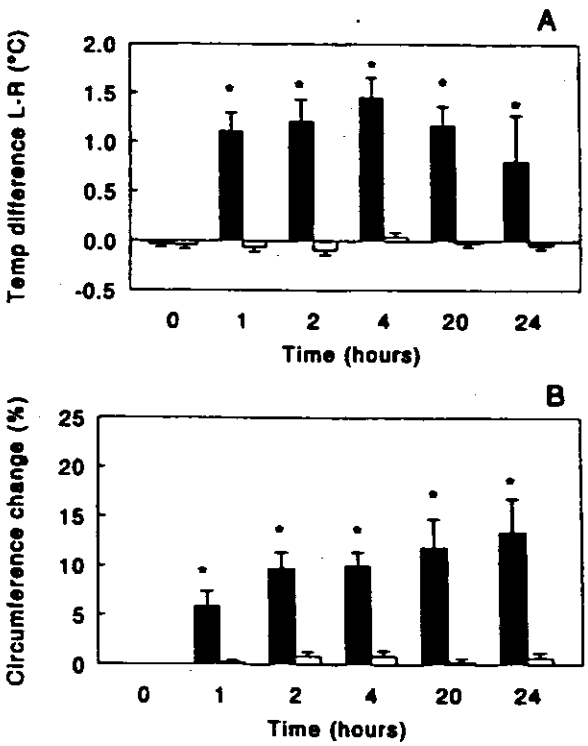

FIGURE 1A Difference in skin temperature between the plantar regions of both hind paws measured pre-operatively (0 h) and during 24 h of continuous infusion. Skin temperature between tert-BuOOH (n = 15): (■) infused paws is compared with normal saline (n = 10): (□) infused paws (* = $p < 0.001$).
FIGURE 1B Percentage change in circumference of the infused hind paw during tert-BuOOH (n = 15): (■) infusion or normal saline (n = 10): (□) infusion (* = $p < 0.01$).

and to both legs of the saline infused animals ($p < 0.001$). Cannulation followed by saline infusion had no effect on skin temperature of the operated hind paw ($p < 0.001$) (Fig. 1A).

The percentual increase of the left foot circumference was 5.9 ± 1.5% 1 h after starting tert-BuOOH infusion ($p < 0.01$) (Fig. 1B). The circumference increased gradually during continuous tert-BuOOH infusion and was 13.4 ± 3.4% after 24 h infusion ($p < 0.01$). Saline infusion did not change the left foot circumference (Fig. 1B). Redness of the plantar side of the left foot was visible after 4 h tert-BuOOH infusion in 90% of the rats, while this phenomenon was not recognizable in the saline infused rats. In 85% of the tert-BuOOH infused rats, redness of the plantar skin was still visible after 24 h infusion.

After 24 h infusion, an impaired function was observed in 66% of tert-BuOOH treated animals, while saline infused rats and the contra lateral leg of the experimental rats demonstrated normal function.

The three different pain observation scores were performed pre-operatively and after 24 h infusion. Comparison of the pain score between tert-BuOOH and saline infused rats was obtained by calculating the difference between the pre-operative and postoperative (after 24 h infusion) value, of both groups. Before the surgical intervention, the spontaneous pain score was 0 in all rats. Infusion with tert-BuOOH induced an increased spontaneous pain score (0.90 ± 0.13) compared to saline (0.02 ± 0.02); ($p < 0.001$). Before the surgical intervention, mechanical stimuli with the Von Frey filaments showed negligible withdrawal reactions. Mechanical stimuli with a 5.16 g Von Frey filament resulted in an increased withdrawal percentage of 40.9 ± 7.1% in the tert-BuOOH group compared to 3.3 ± 4.2% in the saline group ($p < 0.001$) (Fig. 2A). The 12.5 g Von Frey filament induced an increased withdrawal percentage of 54.6 ± 5.3% in the tert-BuOOH group and 6.0 ± 6.0% in the saline control group ($p < 0.001$) (Fig. 2A). Pre-operative observation of the rats on the heated or cold plate showed normal behaviour. Infusion with tert-BuOOH induced an increase of the heat pain score difference to 1.53 ± 0.20 compared to 0.16 ± 0.07 after saline infusion ($p < 0.001$) (Fig. 2B). The cold pain score difference increased to 0.84 ± 0.13 after tert-BuOOH infusion compared to the saline infused animals with a cold pain score alteration of –0.03 ± 0.03 ($p < 0.001$) (Fig. 2B).

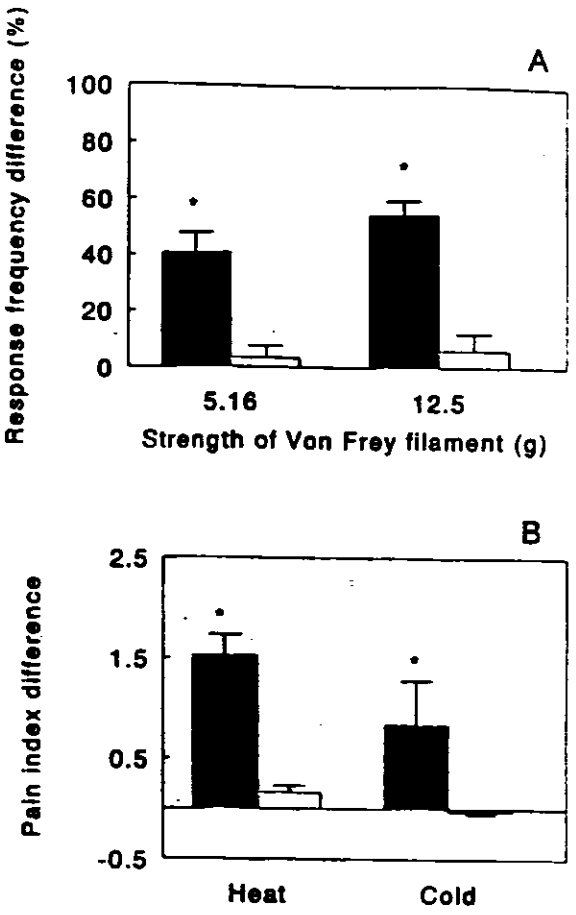

FIGURE 2A Pain in the infused hind limb induced by mechanical stimuli with a Von Frey filament with a force of 5.16 g or 12.5 g. Tert-BuOOH infused (n = 11): (■) and normal saline infused animals (n = 6): (□) (* = $p < 0.001$). Values represent the difference in response frequency observed between pre-operatively and after 24 h infusion.

FIGURE 2B Thermal pain of the infused hindlimb applied by a heated plate (40°C) and cold plate (4°C). The pain index difference is expressed as the difference in pain score observed before and after 24 h infusion. Rats infused with tert-BuOOH (n = 11): (■) are compared with normal saline (n = 6): (□) infused rats (* = $p < 0.001$).

Histology

Light microscopic examination of the left and right gastrocnemius muscles of saline infused animals showed intact, polygonal skeletal muscle fibers, no edema or leukocyte infiltration. Contra lateral gastrocnemius muscles of tert-BuOOH infused extremities were also normal. An infusion period of 4 h tert-BuOOH induced in small regions rounded muscle cells with minimal edema, and without detectable leukocyte infiltration (Fig. 3A). Edema increased after 12 h tert-BuOOH infusion, next to an increased number of rounded muscle cells with less prominent striations (Fig. 3B). The affected areas increased in size and severity as compared to 4 h of tert-BuOOH infusion but unaffected areas were still present. Leukocyte infiltration of the interstitium was not observed after 12 h tert-BuOOH infusion but neutrophilic leukocytes adhering to the endothelial surfaces of the small veins/venules were visible (Fig. 4). After 24 h tert-BuOOH the affected zones were clearly increased as compared to 4 and 12 h infusion (Fig. 3C). Various stages of cellular degeneration were present, consisting of rounding off, decreased striations as well as necrotic muscle cells, distributed in a patchy pattern. Edema, granulocyte infiltration and macrophages were detectable in the affected areas of the gastrocnemius muscle (Fig. 3D). In a pilot study with a lower dose of tert-BuOOH infusion (10 mM, total dose 0.24 mmol), we observed no cellular damage in the left gastrocnemius muscle. The use of a higher dose (100 mM, total dose 2.4 mmol) resulted in severe necrosis in almost the entire gastrocnemius muscle.

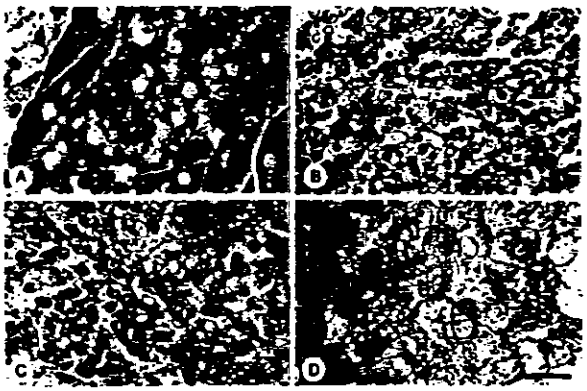

FIGURE 3 Sections of the left gastrocnemius muscle (H.E.-staining). (A) After 4 h of tert-BuOOH infusion small areas with rounded muscle cells (B) after 12 h of tert-BuOOH infusion increased number of rounded muscle cells with increased amount of interstitial fluid (C) after 24 h of tert-BuOOH infusion various stages of cellular degeneration were visible, consisting of rounding off, decreased striations as well as necrotic muscle cells with severe edema (D) detail after 24 h of tert-BuOOH infusion showing muscle cell necrosis, edema and infiltration of neutrophils in the interstitium. Bars = 90 μm (A,B,C); 45 μm (D).

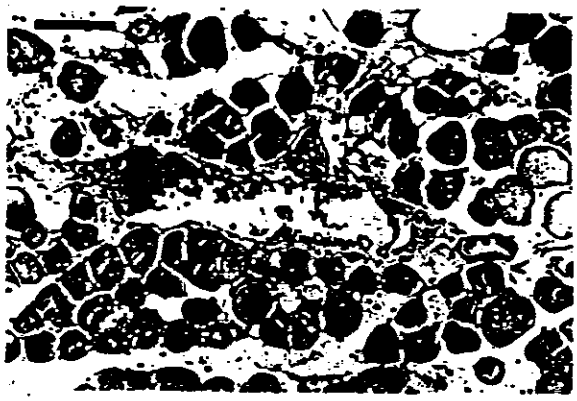

FIGURE 4 Left gastrocnemius muscle (H.E.-staining). After 12 h tert-BuOOH infusion neutrophils adhere to the endothelial surface of a venule. Bar = 45 μm.

Inside the gastrocnemius muscle after 4 h of 25 mM tert-BuOOH infusion small and medium sized arteries seemed unaffected, as observed by light microscopy (Fig. 5A). After 12 h of tert-BuOOH infusion the tunica media of the small arteries showed a reduced amount of the smooth muscle nuclei indicating cell death (Fig. 5B). After a period of 24 h tert-BuOOH infusion necrosis of the medial layers of the vascular wall was apparent; in the transversal sections no nuclei of endothelial cells were present (Fig. 5C).

^{99m}Technetium-IgG Distribution

Scintigrams of 2 rats infused for 24 h with tert-BuOOH imaged within 5 min post injection showed similar uptake of ^{99m}Tc-IgG in the left

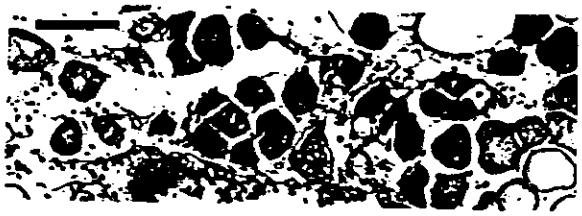

FIGURE 5 Arteriole inside the left gastrocnemius muscle (H.E.-staining) (A) after 4 h tert-BuOOH infusion with intact tunica media (B) after 12 h of tert-BuOOH infusion showing loss of smooth muscle cell nuclei in the tunica media (C) after 24 h tert-BuOOH infusion with necrosis of the vascular wall characterized by disappearance of the smooth muscle cell nuclei. Bar = 22, 5 μm.

paw and right paw (Fig. 6). At 2 h post injection a higher uptake in the left paw was visualized, indicating increase with time due to permeability changes of the capillaries, but unrelated to vascular pooling.

In 24 h saline infused rats (control group) the ^{99m}Tc-IgG uptake ratio of left/right gastrocnemius muscles, established by biodistribution, was 1.05 ± 0.08 (Fig. 7). After 4 h tert-BuOOH infusion, the ^{99m}Tc-IgG ratio of left/right gastrocnemius muscles increased significantly ($p < 0.001$) to 2.09 ± 0.26, with respect to the control group. In animals infused during 12 h with tert-BuOOH a ^{99m}Tc-IgG ratio of 2.04 ± 0.08 was obtained. Infusion of tert-BuOOH during 24 h resulted in a 3.84 ± 0.13 ^{99m}Tc-IgG uptake ratio, a significant ($p < 0.001$) increase compared to

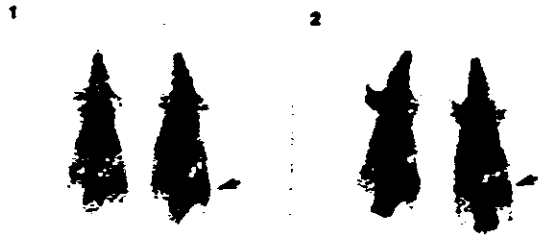

FIGURE 6 Scintigrams of rats (n = 2) after 24 h tert-BuOOH infusion imaged (1) 5 min and (2) 2 h post injection of ^{99m}Tc-IgG. At 2 h postinjection a clearly higher uptake (arrow) of ^{99m}Tc-IgG in the left paw in contrast to the right paw was visualized, indicating a non-flow related increase in vascular permeability.

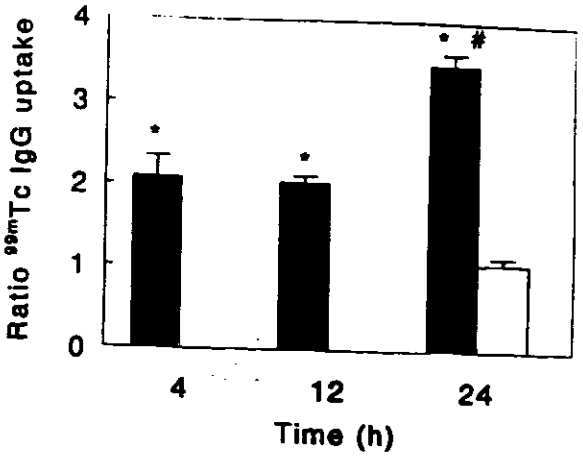

FIGURE 7 Ratio of ^{99m}Tc-IgG uptake in left/right gastrocnemius muscle after 4 h (n = 5), 12 h (n = 5) and 24 h (n = 5) tert-BuOOH infusion (■) or 24 h (n = 3) saline infusion (□). Significantly increased uptake in tert-BuOOH infused versus saline infused (* = $p < 0.001$) hindlimbs, and in 24 h tert-BuOOH infusion versus 4 and 12 h tert-BuOOH infusion periods (# = $p < 0.001$).

the values found after either 4 or 12 h tert-BuOOH infusion or obtained after 24 h saline infusion.

DISCUSSION

This animal model enables to examine the (patho)physiological effects of substances by continuous intra-arterial infusion in non-anaesthetized rats without inducing ischemia. By retrograde placement of the cannula in a small skin artery at the orifice of the femoral artery (microscopically controlled) the arterial circulation of the operated hindlimb remained intact. Absence of ischemia in the operated hindlimb was confirmed by a normal skin temperature of the foot as well as the absence of histological changes in the skeletal muscles of the control group. In contrast to the usual local injection of solutions or infusion directly in the femoral artery in the animal hindlimb, our method offers the possibility to continuously titrate the amount of infusion liquid without essentially disturbing the physiological condition of the animal.

In this study we evaluated free radical induced soft tissue damage in one hindlimb by continuous intra-arterial tert-BuOOH infusion. To our knowledge, an in vivo study of exposing skeletal muscle continuously and intra-arterially with free radicals has not been described so far. The dose of tert-BuOOH used was chosen with regard to a previous study in the rat, which reported that a single intraperitoneal injection of 0.35 mmol tert-BuOOH had no effects on the oxidative defence mechanism of skeletal muscle in the hindlimb.[28] In accordance with these results, we found in a pilot study that 24 h intra-arterial infusion with 10 mM tert-BuOOH (total dose 0.24 mmol) induced no cellular damage or inflammatory reaction, while 100 mM (total dose 2.4 mmol) was immediately followed by severe necrosis of the hindlimb. An intermediate concentration of 25 mM tert-BuOOH infused during 24 h resulted in a final dose of 0.6 mmol, which induced soft tissue damage similar to that observed after ischemia/reperfusion in an extremity.[29] Within 2 h after the onset of tert-BuOOH infusion, signs of inflammation (redness of the skin, increased skin temperature and edema) were recognizable in the infused paw, which persisted during the whole infusion period (Fig. 1). Remarkably, these inflammatory signs were present before extravascular leukocytes were visible in the histological sections (Fig. 3A). In vitro, tert-BuOOH administration to renal tissue induced lipid peroxidation, a decrease in total glutathione content within 15 min and cell death after 30–60 min.[13] In our opinion, the clinical signs of inflammation of the hindlimb as found in this model can only be explained by exposure to free radicals.

Both function and pain observations were performed only after 24 h of infusion to avoid unwanted effects of the anaesthetic agents. Impaired function induced by 24 h of tert-BuOOH infusion (indicated by a shuffling gait) was not accompanied by paralysis of the paw because the withdrawal reaction was still intact.

Pain, one of the symptoms of inflammation, has been assessed in the present study according to various established methods.[20] After 24 h of exposure to free radicals, spontaneous pain-related behaviour was significantly increased in the affected extremity. Increased mechanical sensitivity, as tested by Von Frey filaments, was found in the tert-BuOOH infused hindlimbs; such increased sensitivity has also been reported in arthritic[30] and mononeuropathic rats.[22,31] Thermal stimulation tests are normally performed by means of the thermal struggle test[21] or by measuring the cumulative time-span that the animal removes its foot from the thermal plate.[32] For interpretation of the nociceptive effect of thermal plate exposure, compared to the spontaneous pain behaviour of the rat, we also used the spontaneous pain formula of Attal.[21] The heated plate induced a significantly increased pain behaviour compared to the spontaneous pain behaviour of the tert-BuOOH rat (Fig. 2B). Apparently, free radicals induce allodynia (nociceptive reaction to innocuous stimuli) for a non noxious heat stimulus. In peripheral inflammation, thermal and mechanical allodynia has been associated with the local production of neuroactive inflammatory cytokines and growth factors.[33,34] Free radical initiated soft tissue damage may be followed by a release of cytokines, neuropeptides, by activation of bradykinin receptors resulting in a nociceptive effect. In contrast, on the cold plate tert-BuOOH infused rats exhibited pain behaviour similar to their spontaneous pain behaviour. This non-noxious method for indicating allodynia for cold may not be sensitive enough compared with the thermal struggle test.[21] Possibly, free radical induced soft tissue damage may not be so closely related to cold allodynia.

Histological analysis of gastrocnemius muscles after tert-BuOOH infusion showed various stages of cellular degeneration in combination with accumulation of interstitial fluid. The parenchymal and vascular injury initiated by the free radical donor tert-BuOOH in our animal

model is comparable to ischemia/reperfusion skeletal muscle damage.[29,35–37] The severity of cellular damage and histologically identifiable vascular alterations seem to be dependent on the duration of tert-BuOOH infusion. In the histological sections, neutrophils were absent in the early infusion period, adhered to the luminar surface of the endothelial cells after 12 h, and infiltrated in the skeletal muscle after 24 h of tert-BuOOH infusion. Increased vascular permeability for macromolecules was shown with ^{99m}Tc-IgG after 4 and 12 h of tert-BuOOH infusion. Moreover, after 24 h of tert-BuOOH exposure, the vascular permeability was significantly increased as compared to these earlier periods (Fig. 7). Obviously, early in the infusion period soft tissue damage is directly induced through free radicals without the mediation of leukocytes. Subsequently, additional soft tissue damage could be induced by the continuously exposure to free radicals. Also, the progression of soft tissue damage in combination with severe vascular permeability could be mediated by the neutrophils observed in the skeletal muscle at a later phase. This is also the case in free radical related processes such as ischemia/reperfusion.[8,10,38,39] In our animal model free radicals may upregulate the adhesion molecules on the luminar surface of the endothelial cell, followed by a chemotactic recruitment of circulating neutrophils,[40] which subsequently release proteases and produce even more free radicals.[9,41] Another phenomenon leading to the severe soft tissue damage observed after 24 h of tert-BuOOH could be an edema-induced collapse of the capillaries, followed by tissue ischemia and production of more free radicals.[7] Further evaluation of the progressive soft tissue damage after 24 h of tert-BuOOH infusion (with particular attention to the role of the leukocytes) and the analysis of possible recovery in the affected hindlimb will be performed in future studies. Also, an application for this animal model could be the investigation of the role of antioxidants in soft tissue damage in awake rats in relation to "wholebody" metabolism and function.

In summary, we developed an animal model, allowing the continuous intra-arterial infusion of one hindlimb in non-anaesthetized rats without inducing ischemia. In this model it is possible to evaluate the isolated effects of oxygen-derived free radicals on soft tissue. After initial free radical-related soft tissue damage, increasing necrosis and vascular permeability in skeletal muscle coincide with neutrophilic infiltration.

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