Effect of Short-Term and Long-Term Antioxidant Therapy on Primary and Secondary Ageing Neurovascular Processes

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Previous studies from our laboratory demonstrated an age-related functional decline in sensory neurones and their modulation of microvascular blood flow (primary ageing processes) that correlated with a deficiency in tissue repair (a secondary ageing process). We also raised the notion of a possible role for free radicals in these age-related changes. The aim of this study was to investigate the impact of antioxidant therapy on modulating sensory neurovascular function and tissue repair with age. Twenty-four-month-old Sprague-Dawley rats were treated with vitamin E for short-term (40 mg/kg, i.p., every other day for 2 weeks) or long-term (for 12 months in advance, 10 g/kg, incorporated in food). These treated rats were assessed for the effectiveness of treatment and tested for their sensory neurovascular function, repair of full-thickness burn, and recovery from hyperalgesia following nerve injury. The results indicate that both short- and long-term vitamin E treatments are effective in improving sensory neurovascular function and in reducing the time required for complete wound closure of full-thickness burn injury. Short-term vitamin E treatment was more effective in protecting against the development of hyperalgesia following nerve injury. An initial increase in wound size and in hyperalgesia was observed in the treated animals, and could reflect possible side effects of the antioxidant therapy and support the importance of free radicals in early stages of the repair process. The data, overall, support the notion that oxidative damage contributes to both primary and secondary ageing processes.

Previous complementary studies in our laboratory in rodents and humans have partly attributed the impairment in wound repair with age to a decline in sensory nerve function and a disturbance in its modulation of microvascular blood flow (the sensory neurovascular system) (1,2). Furthermore, emerging evidence from our group and others support the involvement of free radicals in primary (intrinsic) and secondary (pathological) ageing processes involving ageing of the sensory neurovascular system and the consequence delay in tissue repair (3–9).

Different antioxidants including lazaroids, vitamin C, and vitamin E have been illustrated in a number of studies to enhance the healing of wounds by reducing the damage caused by free radicals (10–14). Vitamin E is classified as being the most clinically relevant antioxidant. It is the most effective chain-breaking lipid-soluble antioxidant in the biological membranes, where it contributes to membrane stability (15), cellular and neuronal protection (16), and is known to improve the status of other antioxidants within the body (17).

The link between vitamin E and ageing has received a great deal of attention because vitamin E, via its antioxidant properties (18), may play an important role in reducing age-related decline in physiological functions (19,20) and the risk of several diseases (21–23). Nevertheless, the optimum intake of vitamin E to prevent certain diseases is vigorously debated because of conflicting results obtained from a number of epidemiological studies (24), chemoprevention trials (25,26), and tissue repair studies (27,28).

Research in animals suggests that vitamin E has beneficial effects in wound healing (11,29,30). On the other hand, other studies have shown vitamin E to cause a delay in wound repair (31,32). It is obvious that the use of vitamin E in tissue repair is controversial and warrants further clarification. Furthermore, we previously provided evidence to support the notion that antioxidant therapy could exert both beneficial and harmful effects on tissue repair (3,4). In addition, multiple studies have shown that supplementary intake of antioxidants can lead to poor surgical outcomes, a clinical significance of this study (33,34).

In view of the need to clarify possible beneficial and harmful effects of antioxidant therapy, this study was designed to examine the effect of short- and long-term antioxidant therapy on the function of the sensory neurovascular system and the impact that this might have on tissue repair. In the current study, vitamin E was used at the previously established doses of 40 mg/kg/body wt and 10 g/kg, incorporated in food, for short- and long-term treatment, respectively, because these doses were shown to improve microvascular blood flow (35–38) and improve structural and functional properties of nerves and endothelial cells (39,40), respectively.

Our aim was to study the role of oxidative damage in primary ageing of the sensory neurovascular system and the
impact of this treatment on secondary pathological ageing repair processes using 2 different tissue repair models: (a) a full-thickness burn wound model and (b) a chronically injured sciatic nerve model in old rats. Furthermore, oxidative markers such as total antioxidant status and markers of oxidation of lipids and proteins (malondialdehyde [MDA] and carbonyl protein levels, respectively) were determined to assess the effectiveness of vitamin E in reducing oxidative damage.

METHODS

Young (3 months old) and aged (24 months old) outbred male Sprague-Dawley rats were used. The animals were kept in a plastic cage (2 per cage), in a constant-temperature room (21 \pm 0.5\textdegree C) under a 12/12-hour light/dark cycle, and had free access to food and water. Anesthesia was induced with pentobarbitone sodium (60 mg/kg i.p.). Additional doses of 15 mg/kg were given throughout the experiment to ensure that the rats were kept under anesthesia. This method of anesthesia was previously shown not to alter the basal vasodilator responses (41). The body temperature of the rats was maintained at 37\textdegree C. At the end of each experiment, the animals were killed by barbiturate overdose. All experimental procedures were approved by the Royal Melbourne Hospital Research Foundation Animal Ethics Committee and adhered to International Association for the Study of Pain guidelines.

Vitamin E Treatment

Controls: Young (3 months old) and aged (24 months old) outbred male Sprague-Dawley rats were used. Short-term vitamin E-treated rats: 24-month-old outbred male Sprague-Dawley rats received a natural form of vitamin E (D-alpha-tocopherol succinate) intraperitoneally (40 mg/kg/body wt) every other day for 2 weeks just prior to wound induction or nerve injury. Long-term vitamin E-treated rats: 12-month-old outbred male Sprague-Dawley rats were placed on a vitamin E-rich diet that included 10 g/kg of DL-alpha-tocopherol acetate (synthetic form of vitamin E) in their food for 12 months until the age of 24 months.

Justification of Vitamin E Doses Chosen for Short-Term and Long-Term Treatments

The choice of the dose of vitamin E for short-term treatment (40 mg/kg/body wt) was based on that of Kunisaki’s group, who used this dose since 1994 in all their experimental studies demonstrating an improvement in microvascular blood flow in streptozotocin-diabetic rats (35–38). For long-term treatment, an optimum dose of 1% vitamin E was previously shown to improve structural and functional properties of nerves and endothelial cells in chronic diabetes (39). Van Dam and colleagues (40) demonstrated that administration of a daily supplement of vitamin E (12 g/kg) in food partially prevented nerve dysfunction in young adult streptozotocin-diabetic rats. From those studies there is a consensus that the 2 doses of vitamin E, 40 mg/kg/body wt and 10 g/kg, incorporated in food, used for short- and long-term treatment, respectively, showed improvement in both microvascular and neuronal functions.

Blister Induction and Antidromic Stimulation of Sensory Nerves

A blister was induced in the right hind footpad of the anesthetized rat by applying a suction pressure of \(-40\text{kPa}\) for approximately 30 minutes for young rats and 60 minutes for old rats, using a metal suction cap heated to 40\textdegree C. A small incision was made in the skin at the midthigh region of the anesthetized rat. The sciatic nerve, 1 of the 2 major nerves that innervate the foot, was carefully exposed using blunt dissection, then cut as proximally as possible. The distal portion of the cut nerve was placed over bipolar platinum electrodes and immersed in an oil pool formed from the skin flaps of the wound. The paraffin oil was preheated to 37\textdegree C. The electrodes were fixed in such a position that electrical leakage to adjacent nerve structures was minimized. The surface epidermis of the blister was then removed and a perspex chamber (with inlet and outlet ports) secured over the blister base. Ringer’s solution was perfused over the blister surface continuously during the experiment and maintained at 4 ml/h as in previous experiments (1,42). Activation of the sensory fibers was achieved with a Grass S48 stimulator (Grass Instrument Company, Quincy, MA) at 20 V, 15 Hz, 2 ms square waves, for 1 minute. This set of parameters has been previously used to stimulate sensory C-fibers to induce vasodilatation (43,44). The time elapsed from the start of the blister induction until sciatic nerve stimulation did not exceed 60 minutes for young rats and 90 minutes for old rats.

Measurement of Cutaneous Blood Flow

A laser Doppler flowmeter probe was positioned vertically over the exposed blister in the hindpaw via the perspex chamber. The flux output of the laser Doppler monitor is a function of the concentration and the velocity of the red blood cells moving in the tissue penetrated by the laser light. The changes in relative blood flow (as determined by changes in red cell flux) following electrical stimulation of the cut sciatic nerve were continuously displayed on a chart recorder. Raw data were evaluated by calculating in the area under the stimulation-evoked response curve (area under curve [AUC], cm\(^2\)) for a poststimulation period of 20 minutes. All measurements were made relative to a stable baseline obtained before nerve stimulation. Resultant data were evaluated by calculating the surface area under the stimulation-evoked response curve (cm\(^2\)) for a poststimulation period of 20 minutes of nerve stimulation.

Sodium nitroprusside (SNP, 100 \text{µM}), a directly acting smooth muscle dilator, was perfused in all rats for 10 minutes after baselines were reestablished and used to control for individual variations in smooth muscle reactivity.

Analysis of Blood Flux Response

In each experiment, blood flux responses were measured and normalized by dividing the stimulation-induced area by the ratio of the SNP response divided by the mean nitroprusside response for that group of rats. This correction effectively uses the SNP response as an internal standard for individual variations in the blood flux response to stimulation in each preparation. Statistical evaluation of the
blood flux responses was performed by means of independent Student’s *t* test. A *p* value < .05 was considered significant.

**Tissue Repair Models**

**Thermal burn injury model.**—The fur on the intrascapular region was removed with animal clippers and a cosmetic depilatory agent. At least 24 hours after this treatment, rats were anesthetized with pentobarbitone sodium (60 mg/kg i.p.) and a thermal burn was induced in the treated region using a CO2 laser (50 W power, 2 second duration, and spot diameter of 10 mm). This technique delivers standard energy levels over a given area of skin and therefore gives a similar and reproducible thermal injury (1). This injury results in a large circular wound area (2 cm²). The wound area increases to 2.5–3 cm² by day 2 because of progressive loss of the microcirculation; thermal injury damages capillary endothelial cells. This shape and size of wound allow for healing primarily by contraction and reepithelialization over approximately 3 weeks in old rats. It is also known that round wounds do tend to contract more uniformly, rarely deform into 2 or more wounds, and are associated with less measurement error (45).

**Measurement of healing and statistical analysis.**—The area of burn was traced on a clear plastic sheet daily for the first 6 days and every 48 hours thereafter and measured with a digital planimeter. Measurements were made by an observer who was unaware of the treatment status of the animals. Wounds were left uncovered and observed daily. Any lightly adherent scar was removed with forceps. Although scab formation was minimal, when it occurred, the scab was gently removed. This was done to keep all wounds comparable; previous studies (46) showed that scab formation induces a transient decrease in the rate of wound contraction. It was also easier to accurately measure open-wound area without the overlying scab. The healing endpoint was determined as the time when full wound contraction had occurred. It should be noted that although wound contraction is only one parameter of wound healing, it accounts for large portions of wound closure in full-thickness wounds.

Statistical analyses were performed using a two-way (group × day) repeated-measures analysis of variance (ANOVA), followed by a post hoc Duncan’s pair-wise comparisons. A *p* value < .05 was considered significant.

**Chronic constriction injury model.**—Old rodents were randomly distributed into 4 groups (*n* = 4–6): 3 chronic constriction injury (CCI) (including a control group, short-term, and a long-term vitamin E-treated group) and 1 sham-operated group. Young control rats were randomly distributed into 2 groups: 1 control CCI and 1 sham-operated group. Rats were anesthetized with sodium pentobarbitone (60 mg/kg, i.p.). Body temperature was maintained at 37°C. The production of chronic hyperalgesia was achieved using a modified version of CCI model of Bennett and Xie (47). Briefly, this involved isolating the right sciatic nerve in the mid-thigh region of rats and loosely tying 4 ligatures (4-0 chromic gut) so that they touched, but barely constricted, the nerve. The sham-operated rats, in which the sciatic nerves were isolated but not ligated, were prepared as controls. In all rats, contralateral sides were not disturbed.

After surgery, CCI and sham-operated rats were well groomed and alert without debilitating pain, and could easily remove the paw being tested with noxious stimuli. The animals were kept in a plastic cage (2 per cage) in a constant temperature room (21 ± 0.5°C) under a 12/12-hour light/dark cycle, and had free access to food and water. Nontreated and treated CCI and sham-operated rats were housed in different cages; however, tests were performed on all groups simultaneously.

**Measurement of Thermal Threshold**

Threshold latencies for withdrawal of the hindpaw from a thermal stimulus was measured using a water bath as described by Attal and colleagues (48). The rat was held upright with the head and limb to be tested hanged free, but most of the rest of the body cradled in the hands of experimenter. The paw was then lowered into a water bath kept at 46°C until the rat exhibited a concerned struggle reaction, or 15 seconds has elapsed. Each paw was tested twice, with a 10–15-minute interval between measurements.

Note, ethics approval allowed for continuous monitoring of thermal threshold till normal threshold is achieved (i.e., at 15 seconds) or for a maximum of 10 weeks postinjury.

**Expression of Data and Statistical Analysis**

Changes in thermal hyperalgesia were assessed over time. The differences in thermal thresholds in young and old, sham, and CCI rats, and the effect of saline, short-term, and long-term vitamin E treatments on hyperalgesia were assessed using two-way (group × time) ANOVA.

**Plasma Oxidation Status**

Blood samples were collected from young, old control, and short-term and long-term vitamin E-treated old rats. Plasma was immediately prepared at 800 × g for 20 minutes at 4°C. Total antioxidant status, protein carbonyls, and MDA were measured in plasma. Total antioxidant status was measured using the Randox kit (Randox Laboratories, Crumlin, U.K.), which is based on incubation of 2,2’-azino-bis-3-ethylbenzthiazoline sulphonate (ABTS) with a peroxidase (methmyoglobin) and hydrogen peroxide to produce the radical cation ABTS⁺. This latter species is detected at 600 nm (49). The degree of suppression of the formation of the radicals is proportional to the concentration of antioxidants. Plasma oxidation products were determined in the presence of 50 mM AAPH [2,2’-azobis-(2-aminopropane)-dihydrochloride] at 37°C. AAPH is a water-soluble azo compound that thermally decomposes to generate peroxyl radicals at a constant rate (50). MDA, as a byproduct of lipid peroxidation, was determined by the thiobarbituric acid assay (51). Protein carbonyls, as an estimation of protein oxidation, were measured as previously described (52).
Differences of total antioxidant levels, MDA levels, and protein carbonyl levels between young, old, short-term, and long-term vitamin E-treated plasmas were compared using ANOVA, followed by post hoc Bonferroni’s pair-wise comparison. All results were presented as mean ± SEM (standard error of mean). A *p* value <.05 was considered significant.

**Drugs**

Nembutal (pentobarbitone sodium) was obtained from Boehringer Ingelheim Pty. Ltd. (Queensland, Australia). Chemicals used in Ringer’s solution (9.0 g NaCl, 0.45 g KCl, 0.48 g CaCl₂, 0.2 g NaHCO₃, 1 L H₂O) and vitamin E (d-alpha tocopherol) used for short-term treatment was obtained from Sigma Chemical Co. (St. Louis, MO). Vitamin E (dl-alpha tocopherol) used for long-term treatment was prepared by Ridley Agri Products Pty. Ltd. (Melbourne, Australia).

**RESULTS**

**Effect of Age on the Vascular Response to Electrical Stimulation (ES) in Young and Old Rats**

The ability of the unmyelinated primary afferents in the sciatic nerve to mount a peripheral inflammatory vascular response when stimulated by the selective electrical parameters used, is a reflection of the activity of these afferents. Electrical stimulation of the sciatic nerve at 20 V, 2 ms, for 1 minute at 15 Hz in young rats resulted in a biphasic response—an initial transient drop in blood flow followed by a short-acting vasodilatation response with an AUC of 16.3 ± 1.1 cm². In old control rats, nerve stimulation at this frequency resulted in a significantly reduced vascular response (36% decrease) compared with young controls (AUC: 10.5 ± 1.3 cm²) (Figure 1). There was no significant difference in smooth muscle reactivity between young and old animal groups (data not shown).

**Effect of Age on Healing of Full-Thickness Burns**

The time required to complete wound closure was monitored in young (3 months control) and 2-year-old rats (Figure 2). The initial wound size at day 1 was not different between the 2 groups (young: 1.9 ± 0.2 cm² and old: 1.8 ± 0.2 cm²). Young control rats showed an early increase in wound size that reached a maximum by day 2 then underwent gradual contraction starting from day 3 onwards. Complete wound closure occurred at 14.2 ± 0.3 days. The difference observed in the old control group was a lag phase that extended to 5 days before an actual reduction in wound size started to occur on day 6. In old rats complete wound closure occurred at 21.3 ± 0.3 days.

**Behavioral Observations of CCI in Young and Old Rats**

Rats (young and old) developed behavioral responses 1 to 2 days after induction of CCI. These included abnormal gait, posture, guarding behaviors, and sudden licking of the hindpaw on the side ipsilateral to the ligature of sciatic nerve. Rats were reluctant to place weight on the foot of the injured side. The foot was either raised off the ground and held close to the body or, when used for support, contacted the ground via the medial edge and the heel. The foot itself was markedly ventroflexed, with the toes held tightly together. When raised from the ground by the tail, the rats extended its left hindpaw with the toes spread apart while the hindpaw on the injured side was extended slightly and was held against the scrotum. The abnormal behaviors were obvious up to 3–4 weeks in young and up to 7–8 weeks in old rats.

**Effect of Age on CCI**

The data in Figure 3 show that age had no significant effect on thermal threshold in sham-operated rats. We also showed that induction of CCI in both young and old rats causes a similar reduction in thermal threshold 1–4 weeks after injury with thermal threshold at week 4, reaching 8.9 ± 0.2 seconds in young and old CCI rats, respectively. However, in subsequent weeks, the thermal thresholds in young CCI rats were significantly different from old CCI rats, reaching 10.9 ± 0.3 seconds and 8.7 ± 0.2 seconds for young and old rats at week 5, 11.8 ± 0.4 seconds and 8.8 ± 0.2 seconds for young and old rats at week 6, and 13.9 ± 0.3 seconds and 8.5 ± 0.2 seconds for young and old rats at week 7. In week 8, full recovery with a return to normal threshold was achieved in young rats (14.7 ± 0.2 s) compared with old rats (8.7 ± 0.2 s). Old rats did not show any sign of recovery until weeks 9 and 10, where thermal thresholds increased to 9.9 ± 0.3 and 11.2 ± 0.4 seconds, respectively, but complete recovery was not achieved within the 10-week period of observation permitted by the Animal Ethics Committee.

**Effect of Age on Plasma Oxidation Status**

Total antioxidant status was assessed in plasma collected from young and old rats (Figure 4A). There was a significant decrease of 28% in total antioxidant status in aged controls (1.2 ± .07 mM) compared with young controls (1.7 ± .05 mM).

Measurements of lipid peroxidation product, MDA (Figure 4B), and protein oxidation product, protein carbonyls (Figure 4C), in plasma at basal levels (in absence of the
free radical generator, AAPH) and during oxidative stress (in the presence of AAPH) showed a similar profile.

In the absence of AAPH, a small but insignificant increase of MDA formation was observed in old rats (4.6 ± 0.16 μmol/L) compared with young rats (3.8 ± 0.13 μmol/L). However, under oxidative stress (in the presence of AAPH), a significant increase of 39% of MDA was formed in the plasma of old rats (13.3 ± 0.37 μmol/L) compared with young rats (9.6 ± 0.04 μmol/L) (Figure 5B).

Furthermore, at basal levels, a significant 38% increase of protein carbonyls was observed in old rats (0.33 ± 0.006 nmol/L) compared with young rats (0.24 ± 0.004 nmol/L), and when the plasma was exposed to AAPH (under oxidative stress), a further significant increase of 21% of protein carbonyls was observed in old rats (0.40 ± 0.005 nmol/L) compared with young rats (0.33 ± 0.007 nmol/L) (Figure 4C).

Effect of Short-Term and Long-Term Vitamin E Treatment on Plasma Oxidation Status in Old Rats

Total antioxidant status in the plasma collected from old, short-term, and long-term vitamin E-treated rats is illustrated in Figure 5A. Old rats treated with either short- or long-term vitamin E treatment showed a marked significant increase of 63% and 71%, respectively (2.0 ± 0.04 and 2.1 ± 0.04 mM, respectively), in their antioxidant status compared with old controls (1.2 ± .07 mM). Supportive evidence for this improved antioxidant status of the treated rats was obtained by measurement of MDA (Figure 5B) and protein carbonyl (Figure 5C) formation in plasma at basal levels (absence of AAPH) and during oxidative stress induced by the free radical generator AAPH.

Both short- and long-term vitamin E-treated rats showed a significant decrease of 31% and 38% in MDA formation at basal levels (absence of AAPH) (3.2 ± 0.06 and 2.8 ± 0.04 μmol/L, respectively) compared with their own controls (4.6 ± 0.16 μmol/L). In addition, a significant 30% and 31% reduction during oxidative stress (presence of AAPH) (9.4 ± 0.13 and 9.1 ± 0.16 μmol/L, respectively) compared with aged controls (13.3 ± 0.35 μmol/L) in MDA levels was also observed.

Protein carbonyls at basal level was significantly decreased by 32% and 36% in both short- and long-term vitamin E-treated rats (0.23 ± 0.01 and 0.21 ± 0.01 nmol/L), respectively, compared with old controls (0.33 ± 0.01 nmol/L). Both short- and long-term vitamin E-treated rats also showed a significant reduction of 37% and 34%, respectively, in protein carbonyl levels (0.25 ± 0.01 and 0.26 ± 0.01 nmol/L, respectively) compared with old control rats (0.40 ± 0.01 nmol/L) during oxidative stress (in the presence of AAPH).

Effect of Short-Term and Long-Term Vitamin E Treatment on the Vascular Response to Electrical Stimulation in Old Rats

Old rats treated with short-term vitamin E showed a significantly enhanced response of 181% (AUC: 29.0 ± 3.2 cm²) compared with aged controls (AUC: 10.5 ± 1.3 cm²) (Figure 6). There was also a significant increase of 81% in the vascular response to electrical stimulation of long-term vitamin E-treated rats (AUC: 19.0 ± 3.7 cm²) compared with aged controls (AUC: 10.5 ± 1.3 cm²) (Figure 6). No difference was observed in smooth muscle reactivity between the 3 animal groups (data not shown).

Effect of Short-Term and Long-Term Vitamin E Treatment on Healing of Full-Thickness Burns in Old Rats

The effect of short- and long-term vitamin E treatment at 40 mg/kg/body wt i.p. for 2 weeks every other day and 10 g/kg, incorporated in food, for 12 months, respectively, on healing of full-thickness burns in old rats is shown in Figure 7. The initial wound size at day 1 was not different between the 3 groups (old: 1.8 ± 0.2 cm², short-term treated: 2.1 ± 0.1 cm², long-term treated: 2.2 ± 0.1 cm²). Both groups of old rats receiving short- and long-term vitamin E showed a very similar profile of wound healing that was unique in comparison to the old control rats. Both treated groups
showed a significant increase in wound size on day 4 (4.4 ± 0.3 cm² and 3.7 ± 0.2 cm², respectively) and day 6 (3.9 ± 0.2 cm² and 3.4 ± 0.1 cm², respectively) compared with old controls (2.7 ± 0.2 cm²) and (2.4 ± 0.3 cm²) for day 4 and 6, respectively. The lag phase of the treated groups extended till day 7 as opposed to day 5 in old controls, after which wound contraction started to occur. There were no differences in wound sizes between the 3 groups during days 8–14. However, both short-term and long-term vitamin E-treated rats showed accelerated wound closure over days 14–16, with complete wound closure occurring at 16.5 ± 0.3 or 16.0 ± 0.4 days, respectively, compared with the aged controls, where complete wound closure was achieved by 21.3 ± 0.3 days (Figure 7).

**Effect of Short-Term and Long-Term Vitamin E Treatment on Recovery From Nerve Injury in Old Rats**

Old rats that received long-term vitamin E treatment showed a decrease in their thermal sensitivity (8.0 ± 1.2 s) compared with control rats (9.9 ± 0.2 s) at week 1 postinjury; however, it did not reach statistical significance. On the other hand, short-term vitamin E-treated rats showed a smaller decrease in their thermal sensitivity (12.4 ± 1.1 s) that was less than other CCI groups including control CCI and long-term vitamin E CCI-treated rats (Figure 8). Old CCI controls showed a steady but a nonsignificant decline over weeks 2–8. However, both treated old groups showed a different profile with an initial deeper decline in thermal sensitivity that lasted for weeks 2–5 in long-term vitamin E-treated rats, and only for week 2 in short-term vitamin E-treated rats. Old control CCI rats showed an improvement in thermal sensitivity by week 9 (9.9 ± 0.3 s). However, by the end of the monitoring period at week 10, the thermal threshold was still significantly reduced (11.2 ± 0.4 s) compared with sham levels (15 ± 0.1 s; see Figure 3). Old short-term vitamin E CCI-treated rats showed signs of recovery by week 3 and week 4, where thermal threshold reached 12.2 ± 0.5 and 13.1 ± 0.6 s, respectively, and recovery was completed by week 5 (14.7 ± 0.2 s). Old rats treated with long-term vitamin E showed signs of recovery by week 7 (9.8 ± 0.9 s) and 8 (10.9 ± 0.9 s) and the thermal threshold was approaching normal by week 10 (14.6 ± 0.2 s).

**DISCUSSION**

Previous studies from our laboratory demonstrated an age-related functional decline in sensory neurones and their modulation of microvascular blood flow (a decline in sensory neurovascular function) that correlated with a deficiency in repair efficacy (1,2,9). It is evident, therefore, that factors that diminish sensory nerve function with age will ultimately contribute to alteration in microvascular blood
flow and delayed tissue repair (1). In this study and in support of our previous findings (9), we demonstrated a decline in aged sensory nerve response to electrical stimulation (Figure 1). The concept that the decline in sensory nerve function with age contributes significantly to poor healing qualities of tissues in aged rats was also previously investigated in our laboratory using a full-thickness skin burn wound model (1) and a neuropathic pain model (chronic constriction nerve injury model) (4,53). In this study, we provided new sets of data that confirmed age-related delay in tissue repair using the above-mentioned studies, which used the thermal wound model (Figure 2) and the chronic constriction nerve injury (CCI) model (Figure 3).

Our previous studies (3,4) in injured aged animals regardless of the type of injury (skin or nerve injury) were suggestive of a major role for oxidative damage in delayed tissue repair with advancing age. In support of this proposition, the current study shows an age-related decrease in the antioxidant status accompanied by an increase in plasma levels of lipid peroxidation and protein oxidation both before and after oxidative stress challenge (Figure 4A–C). The observation of delayed tissue repair and the increase in oxidative damage with age merits the use of antioxidant therapy to improve the redox status of aged rats, which in theory should preserve or improve the function of the sensory neurovascular system and hence improve tissue repair.

Improved antioxidant status following antioxidant supplementation is well documented (54–57), and can be reflected by decreased lipid peroxidation (58–60) and protein oxidation (61,62). Our study provided evidence that both short- and long-term vitamin E treatment resulted in improvement in antioxidant status (Figure 5A) and reduction in levels of lipid peroxidation and protein oxidation both before and after oxidative stress challenge (Figure 5B and C). This was not surprising in view of the fact that these oxidative stress measures only reflect changes in short-term oxidative damage rather than cumulative damage over a long period of time. Nonetheless, that these measures were significantly altered by both treatments is indicative of the effectiveness of both short- and long-term treatment with vitamin E.

Furthermore, both short- and long-term vitamin E treatment of old rats significantly improved microvascular blood flow in response to electrical stimulation by 181% and 81%, respectively, compared with old controls (Figure 6). The data may indirectly indicate that vitamin E protects the neurons and/or the microvasculature from getting attacked by free radicals, thus preserving the physiological and functional integrity of nerve cells. This is in agreement with other studies where improvement of microvascular blood flow in diabetes was demonstrated using vitamin E (35–38). It could be postulated that vitamin E may have a direct effect on endothelial and/or smooth muscle cells, and its protective effect...
stimulation of the sciatic nerve at 20 V, 2 ms, for 1 minute at 15 Hz in old rats. Body wt i.p. for 2 weeks every other day and vitamin E at 10 g/kg, incorporated in food for 12 months, respectively) on the vascular response to electrical stimulation of the sciatic nerve at 20 V, 2 ms, for 1 minute at 15 Hz in old rats. Both short- and long-term vitamin E-treated rats showed accelerated wound closure (16.5 ± 0.3 or 16 ± 0.4 days, respectively) compared with aged controls (21.3 ± 0.3 days). However, it was noticeable during the first 5 days post wound induction that both treated groups showed a significant increase in wound size compared with control. * Denotes significant difference from the control group (p < .05) (n = 5–6).

It was anticipated that the improvement in the sensory neurovascular system in the treated rats as observed in Figure 6 would result in improvement in tissue repair processes of the full-thickness burn wound and injured sciatic nerve (CCI model).

The current study showed that old rats receiving either short- or long-term vitamin E treatment exhibited a longer lag phase of around 7 days as opposed to 5 days (observed in control rats) before wound contraction started to occur (Figure 7). This negative effect of antioxidant therapy on the early stages of wound repair that is characterized by inflammatory processes could be related to vitamins E’s anti-inflammatory action, which stems from its ability to inhibit phospholipase-A activity and thus the production of prostaglandins (67). Since adequate inflammatory processes are essential prerequisites for tissue repair, the anti-inflammatory action of vitamin E could be harmful during the early stages of the repair process. Alternatively, this negative effect may be related to vitamin E’s free radical scavenging property, as free radicals produced by neutrophils aid in cellular defense. This proposition is further supported by a study by Teo and Naylor (68), which used a wound contraction animal model treated with allopurinol, a free radical suppressor, and showed that inhibiting free radical formation could attenuate the inflammatory response and fibroblast–myofibroblast transformation. This proposition could provide an explanation for the early negative effect of vitamin E on wound size observed in this study, and supports our previous study where we showed that free radicals exert a positive effect during the early stages of tissue repair (4).

Following the inflammatory stage of wound repair, the early proliferative phase takes over, with angiogenesis as a major component (69). As shown in Figure 7, there were no differences in wound size between the 3 groups during days 8–14. This could be due to vitamin E scavenging of 2 oxidants with an opposing effect on angiogenesis. It has not change with age (63–66). In support of this notion, the vasodilator response to SNP was not different in young and old rats (data not shown) and in the treated and nontreated rats, inferring that there was no change in smooth muscle reactivity in the vitamin E-treated rats. This suggests that the enhancement of the microvascular blood flow is independent of changes in vascular smooth muscle reactivity. We concede, however, that possible direct effect of vitamin E on endothelial function could be a contributing factor to the overall improvement observed in the function of the sensory neurovascular system.

It was interesting to observe that old rats that received long-term vitamin E treatment showed an 81% increase in their sensory neurovascular response compared with its own control, an increase that was not as high when compared with old short-term-treated rats (181%). It was anticipated that the protective effect of long-term vitamin E treatment would be greater than that of short-term vitamin E treatment. The fact that both 2 weeks and 12 months of vitamin E treatment improved the sensory neurovascular function to a level equivalent to young animals (long-term treatment) or higher (short-term treatment) could be indicative that the effect of oxidants in modulating this function is temporary and reversible. That short-term treatment induced a greater enhancement of the response is surprising and any proposed mechanism for this enhancement is purely speculative. It could be argued that long-term vitamin E treatment over 12 months resulted in a balanced redox status in old animals (similar to young) and consequently resulted in preservation of the normal functioning of the sensory neurovascular system. In contrast, short-term treatment could have resulted in a sudden increase in the antioxidant defense mechanisms, overcoming a prolonged inhibitory oxidative effect resulting in a rebound effect with the outcome of an enhancement over and above normal responses.

Since a major part of tissue repair is dependent on an intact sensory innervation and adequate microcirculation, a level equivalent to young animals (long-term treatment) or short-term vitamin E treatment would be greater than that of short-term vitamin E treatment. This suggests that the protective effect of long-term vitamin E treatment improved the sensory neurovascular function to a level equivalent to young animals (long-term treatment) or higher (short-term treatment) could be indicative that the role is not entirely due to the maintenance of neuronal function. However, previous investigations including our own have reported that smooth muscle cell function does not change with age (63–66).
been shown that the superoxide anion generated at the wound site inhibits angiogenesis (70), whereas hydrogen peroxide enhances angiogenesis (71). The outcomes of the competition between the opposing effects of the 2 oxidants may have cancelled each other out, which in turn could reflect the lack of difference between the treated and untreated groups during this early proliferative phase. It is important to note here that the absence of an observed difference between treated and nontreated groups in the early proliferative phase does not reflect inaction of the antioxidant therapy during the overall proliferative phase. Indeed the impact of vitamin E therapy became apparent during the late proliferative phase that includes contraction and reepithelization. Both short- and long-term vitamin E-treated rats showed accelerated wound closure over days 14–16 with complete wound closure occurring 16.5 ± 0.4 or at 16 ± 0.6 days, respectively, compared with aged controls where complete wound closure was achieved by 21.3 ± 0.3 days (Figure 7). We propose that protection against tissue peroxidation may have improved collagen disposition in the treated rats and hence accelerated wound contraction. This is supported by a previous study showing collagen to be denatured by hydroxyl radicals (72). It is also possible to postulate that vitamin E could have inhibited the increased glycosylation of tissue proteins (73), which is known to contribute to impaired contraction of granulation tissue during wound repair (74). Thus the positive enhancing effect of tissue repair observed in the treated rats can be partly attributed to vitamin E’s protective effect, preserving collagen function and inhibiting advanced glycation end-products.

Previous studies in our laboratory showed that short-term acute treatment with antioxidants resulted in a nonsignificant reduction in hyperalgesia but did not accelerate the recovery of injured nerves in young CCI rats (3). In that same study, we have also shown that nerve injury in old rats resulted in a significantly greater oxidative stress status compared with young animals. In the current study, we provide novel evidence that short-term vitamin E-treated rats showed a significantly smaller reduction in their thermal sensitivity compared with old CCI controls and long-term vitamin E-treated rats and a significantly shorter course of injury (Figure 8). As discussed previously, this may be due to the de nova availability of vitamin E in the body of rats that received short-term vitamin E, which may have resulted in a sudden increase in the antioxidant defense mechanisms overcoming a prolonged inhibitory oxidative effect.

As shown in Figure 8, a steady but insignificant decline in thermal sensitivity over weeks 2–8 was shown by old CCI rats. However, both treated old groups showed a different profile with an initial deeper decline in thermal sensitivity shown in weeks 2–5 in long-term vitamin E-treated rats and only for week 2 in short-term vitamin E-treated rats. This indicates a detrimental effect of long-term vitamin E treatment in the initial stages of neuronal repair.

By the end of the monitoring period at week 10, old CCI rats did not fully recover as their thermal threshold remained significantly lower than normal thresholds in sham animals. Short-term treatment with vitamin E not only reduced the extent of thermal hyperalgesia associated with neuronal injury but also accelerated recovery with a return to basal thermal thresholds by week 5 postinjury. However an alleviation of thermal hyperalgesia was seen in long-term vitamin E rats starting only at week 5 and 6, but thermal threshold was still lower than old control CCI, and only by week 7 that improvement above control was observed with thresholds approximating normal levels by week 10. Despite the initial inhibitory effect exerted by vitamin E in the early stages of nerve repair compared with old control CCI rats, accelerated nerve recovery still occurred in long-term vitamin E-treated rats at week 10 with their thermal threshold reaching 14.6 ± 0.2 seconds compared with a threshold of 11.2 ± 0.4 seconds for control CCI rats.

The anti-inflammatory properties of vitamin E could explain the inhibitory effect exerted by vitamin E in the early stages of nerve repair as previously alluded to in the wound repair model. Furthermore, the protective effect exerted by vitamin E in the later stages of nerve repair could be attributed to its free radical scavenging property. It is well known that oxidants including nNO are known to increase with age (75,76) and contribute to maintenance of hyperalgesia due to nerve injury (53,77,78), and that nNO contributes to delayed recovery of injured nerves in old rats and to the maintenance of thermal hyperalgesia with age (3). It was suggested that this effect is mediated by peroxynitrite resulting from the interaction of superoxide anions and NO (3). The proposition that accelerated recovery in vitamin E-treated rats could be related to its ability to reduce peroxynitrite formation by Beharka and colleagues, who showed that vitamin E has a combined effect in reducing NO and superoxide anion, leading to decreased peroxynitrite formation in old mice (79). Overall, the data in Figure 8 suggest that short-term treatment with vitamin E at the dose of 40 mg/kg appears to be an effective neuroprotective agent, and that long-term treatment with vitamin
E could have an early negative effect (enhancing hyperalgesia) and a late positive effect (accelerating recovery of injured neurones).

Conclusion

This study provided evidence to support our previous reports of a decline in the sensory neurovascular function with age combined with a delay in tissue repair. We provided novel data to suggest that the imbalance between the oxidative damage and the local antioxidant defense system in old rats could be a contributing factor to this decline. The results indicate that both short- and long-term vitamin E treatment are equally effective in reducing oxidative stress markers in the plasma of old rats. The current data also showed both short- and long-term vitamin E treatments to be effective in improving sensory neurovascular function and in reducing the time required for complete wound closure of full-thickness burn injury. However, it was evident from the results that short-term vitamin E treatment was more effective than long-term vitamin E treatment in protecting against the development of hyperalgesia subsequent to nerve injury. A note of caution is that the initial increase in wound size in both short- and long-term-treated groups, and the initial increase in hyperalgesia in CCI animals in the long-term treated group, could reflect a possible side effect of the antioxidant treatment, supporting the notion that free radicals are essential for early stages of the repair process. The data, overall, support the notion that oxidative damage contributes to both primary and secondary ageing processes.

Acknowledgment

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