THE JOURNAL OF BONE & JOINT SURGERY (Br)

Oral vitamin C attenuates acute ischaemia-reperfusion injury in skeletal muscle

S. R. Kearns, D. Moneley, P. Murray, C. Kelly, A. F. Daly

From Cappagh Hospital and Beaumont Hospital, Dublin, Ireland

Ischaemia-reperfusion injury (IRI) is caused by endothelial and subendothelial damage by neutrophil-derived oxidants. Vitamin C is an antioxidant which attenuates endothelial injury after IRI. Our aim was to evaluate the effect of oral vitamin C in the prevention of IRI in skeletal muscle. We used a model of cross-clamping (3 hours) and reperfusion (1 hour) of the cremaster muscle in rats. Muscle function was assessed electrophysiologically by electrical field stimulation. Infiltration by neutrophils was determined by the activity of tissue myeloperoxidase (MPO) and tissue oedema by the wet-to-dry ratio. Neutrophil respiratory burst activity was measured in control animals and groups pretreated with vitamin C.

IRI significantly decreased muscle function and increased muscle neutrophil MPO activity and muscle oedema. Pretreatment with vitamin C preserved muscle function and reduced tissue oedema and neutrophil infiltration. Neutrophil respiratory burst activity was reduced in the group treated with vitamin C compared with the control group.

We conclude that pretreatment with oral vitamin C protects against acute muscle IRI, possibly by attenuating neutrophil respiratory burst activity.

J Bone Joint Surg [Br] 2001;83-B:1202-6.
Received 26 September 2000; Accepted after revision 16 May 2000

S. R. Kearns, AFRCS I, Specialist Registrar in Orthopaedics
James Connolly Memorial Hospital, Blanchardstown, Dublin 15, Ireland.

D. Moneley, AFRCS I, Registrar in General Surgery
P. Murray, FRCS Orth, Consultant Orthopaedic Surgeon
C. Kelly, MCh, Professor of Vascular and General Surgery
Beaumont Hospital, PO Box 1297, Beaumont Road, Dublin 9, Ireland.

A. F. Daly, MRCP I
Faculty of Health Sciences, Trinity College, Dublin 2, Ireland.

Correspondence should be sent to Mr S. R. Kearns.

0301-620X/01/811754 $2.00

Temporary interruption of vascular patency followed by the reintroduction of blood flow causes a specific pattern of tissue damage termed ischaemia reperfusion injury (IRI). Clinically, it is responsible for tissue necrosis after arterial thrombosis/revascularisation such as occurs in myocardial infarction and embolic limb injury. For orthopaedic surgeons, IRI is of particular interest since it can occur in skeletal muscle distal to a pneumatic tourniquet or secondary to tissue oedema and subsequent fasciotomy in compartment syndrome.

Tissue ischaemia and reperfusion cause profound injury to local and systemic tissues via a predominantly neutrophil-mediated inflammatory cascade.[1-4] Endothelial dysfunction occurs as a result of tissue hypoxia and on subsequent reperfusion, cytokines and adhesion molecules are enhanced leading to chemotaxis and infiltration by neutrophils. Necrosis of damaged tissue by neutrophil myeloperoxidase (MPO) and other enzymes generates active oxygen species, which in turn can extend the ischaemic injury.

Studies have confirmed the key role of neutrophilic infiltration in IRI.[1-3] Depletion of the numbers of neutrophils by haemofiltration or adhesion by immunological means can moderate IRI to a considerable extent.[5-8] Similarly, in various models of IRI, the administration of antioxidants limits the injury caused by neutrophil-derived oxygen free radicals, including systemic injury after ischaemia of the lower torso.[9,10] Vitamin C is a water-soluble endogenous antioxidant which acts by scavenging aqueous-phase reactive oxygen species by very rapid electron transfer.[11] Supplementation with vitamin C has been shown to protect against oxidant-mediated endothelial injury in vivo.[9]

Our aim was to investigate whether pretreatment with oral vitamin C attenuated acute IRI in skeletal muscle. We used a model of clamping and reperfusion of the cremaster neurovascular pedicle in rats. This allows the isolation of an intact skeletal muscle on its neurovascular pedicle and assessment of muscle function and of neutrophil-mediated muscle injury.

Materials and Methods

The study consisted of two sections. The first assessed the effect of oral vitamin C on IRI-induced changes in weight

(oedema), the functional status and the MPO activity of the cremaster muscle. The second section examined the effect of vitamin C on respiratory burst activity in whole blood.

Experiment 1

Animal preparation. We randomised 18 Sprague-Dawley rats (300 to 400 g) to one of three groups: control + normal diet, IRI + normal diet, IRI + vitamin C. The animals in the last group received 2 g of vitamin C per kg per day (Roche Pharmaceuticals, Clonskeagh, Dublin, Ireland) for five days administered in 30 ml of drinking water. The oral dose of vitamin C was selected after reviewing previously published regimens and their efficacy in animal and human models at doses of from 200 mg to 3 g/day.[11-15]

Isolation of the cremaster muscle,[16] cross-clamping and reperfusion. The animals were anaesthetised with inhalational halothane and placed supine on the table. The temperature was maintained by a heating lamp and monitored using a rectal thermometer. We made a longitudinal incision in the skin and fascia, of 1 to 2 cm, on the ventral aspect of the right scrotum. The remaining connective tissue was carefully separated by blunt dissection from the cremaster sac and the muscle opened with microsurgical scissors on the less vascular ventral plane, taking care to avoid the larger anastomosing vessels.

The main testicular blood vessels were freed by dissecting the connective tissue proximally, down to the end of the muscle pouch. The vessels between the testis and the cremaster muscle were cauterised and divided. The mesoepididymis and testicular vessels were tied off and divided with scissors, freeing the testis and epididymis, which were discarded. The neck of the cremaster muscle was incised circumferentially to isolate the muscle completely except for a pedicle containing the cremaster neurovascular bundle. The isolated pedicle was cross-clamped for three hours with a microvascular clamp, followed by reperfusion for one hour. The muscle was divided and samples were collected for assessment of muscle function, weight and neutrophil MPO activity. On completion of the experiment the animals were killed by a lethal intracardiac dose of sodium pentobarbitone.

Assessment of muscle function - electrical field stimulation. We identified and isolated a strip of muscle 2.5 × 0.5 cm in size from a standard central area. A 4/0 silk suture was secured to one end in situ and the muscle strip was freshly excised. The other end was fixed to an isometric force transducer by a tissue clip (Radnoti Glass Technology Inc, California). The muscle strip was mounted, with its long axis in the vertical plane and suspended between two platinum electrodes set vertically, approximately 1 cm apart (Radnoti Glass Technology Inc). It was then lowered into a 50 ml water-jacketed glass tissue chamber (Radnoti Glass Technology Inc). The transducer was fixed to a retort stand via a transducer micropositioner (Radnoti Glass Technology) which allowed adjustment of the preload tension on the muscle. It was used in conjunction with a built-in amplifier with an effective range of 0 to 20 g. The cremaster muscle strip was maintained in a bicarbonate buffer solution (Sigma Chemical Co Ltd, Irvine, UK) and the pH was corrected to 7.3 to 7.4. The pH and O_2 saturation of the solution were maintained by constant aeration with a 95%O_2/5%CO_2 gas mixture (BOC Gases, Dublin, Ireland). The temperature of the chamber was maintained at 37°C throughout the experiment by circulating warm water through the outer jacket (Cole Parmer Immersion Circulator; Foss Electric, Dublin, Ireland).

The muscle was stimulated along its whole length by bipolar platinum electrodes. Supramaximal pulses (20 V, 2 msec square-wave duration[17-19] and 40 Hz) were obtained from a pulse generator (Harvard stimulator; Harvard Apparatus, Kent, UK). The muscle length was adjusted to the optimal length of the fibre bundles, i.e., that which produced maximal isometric force, and electrical field stimulation was performed. The isometric contraction of each muscle strip was assessed in response to a timed series of twitch and tetanic electrical stimuli. The muscle strip was then weighed (Oertling YA124 Analytical Balance; Avery Berkel, Warley, UK).

Myeloperoxidase assay. MPO is a haem-containing enzyme stored in the azurophil granules of neutrophils. Its measurement has been shown previously to be a simple method of quantitative assessment of tissue neutrophil sequestration.[20] After weighing, a section of the cremaster muscle was homogenised in 10 ml of 20 mM potassium phosphate buffer (pH 7.4) containing 0.1 mM EDTA; 2 ml were then centrifuged at 20 000 *g* for 15 minutes at 4°C to pellet the insoluble cellular debris. The resultant supernatant which contained < 5% of total MPO activity and > 95% of the water-soluble haemproteins (myoglobin and haemoglobin) was discarded. The pellet was rehomogenised in an equivalent volume of 0.05M potassium phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide. The sample was then freeze-thawed twice.

The samples were assayed spectrophotometrically (Beckton-Dickinson, Mountain View, California) for activity of MPO by incubating 10 μl of homogenate with 290 μl of a solution containing 2.9 ml of O-dioniside dihydrochloride in 90 ml of distilled water, 10 ml of 50 mmol/l potassium phosphate buffer (pH 6) and hydrogen peroxide. The change in absorbance with time was then measured at 450 nm. One unit of MPO was defined as that which degraded 1 mol of hydrogen peroxide per minute at 25°C.

Wet-to-dry ratio. The wet-to-dry ratio is a simple assessment of tissue oedema. A separate section of freshly harvested cremaster muscle was weighed (Oertling YA124 Analytical Balance) and then heated at 60°C in a gravity convection oven (Model IH-150, Sanyo Gallenkamp Plc, Loughborough, UK) for 72 hours until the weight had become constant. The difference between the fresh and dried samples was recorded.

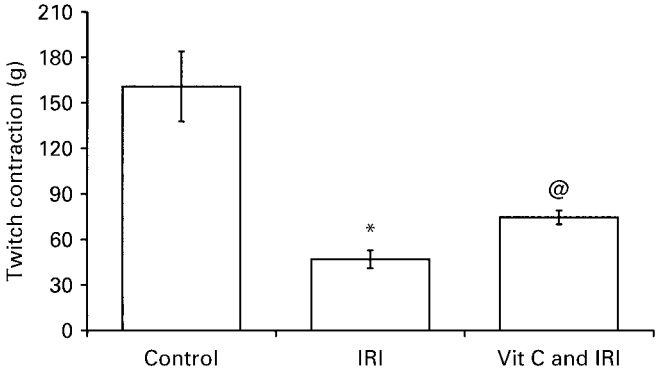

Fig. 1

Effect of IRI on the mean (±SEM) muscle peak twitch contraction (*p < 0.01 *v* control, @ p < 0.05 *v* IRI (ANOVA)).

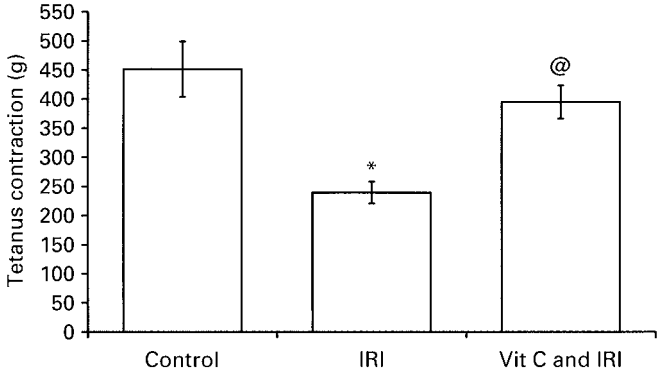

Fig. 2

Effect of IRI on the mean (±SEM) muscle tetanic contraction (*p < 0.01 *v* control, @ p < 0.05 *v* IRI (ANOVA)).

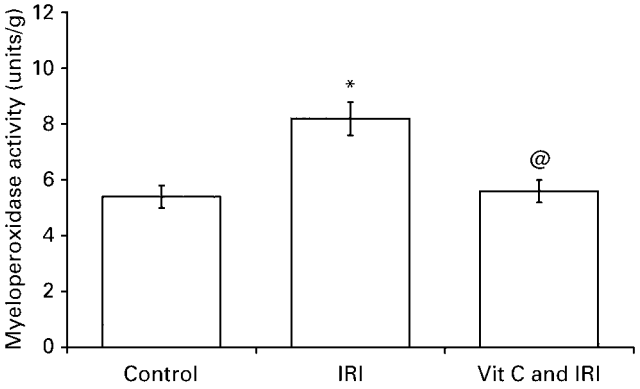

Fig. 3

Effect of IRI on the mean (±SEM) muscle neutrophil infiltration (p < 0.01 *v* control, @ p < 0.05 *v* IRI (ANOVA)).

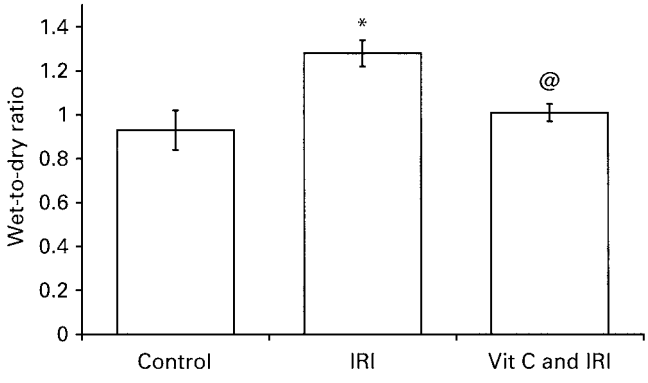

Fig. 4

Effect of IRI on the mean (±SEM) muscle wet-to-dry ratio (*p < 0.01 *v* control, @ p < 0.004 *v* IRI (ANOVA)).

Experiment 2

In the second experiment 12 animals were randomised either to a normal diet or normal diet and supplemental vitamin C (2 g per kg per day for five days). The animals were killed on day 5 and whole blood taken for estimation of respiratory burst activity.

Neutrophil respiratory burst activity. Blood was taken by cannulation of the right ventricle with a 25-gauge needle and assayed within four hours. The respiratory burst was assessed using a Bursttest (Orpegan, Heidelberg, Germany). This method allows the determination of the oxidative and enzymatic activity of leukocytes using dihydrorhodamine 123 (DHR 123) as a fluorogenic substrate, and measures flow cytometrically.[21] Whole blood (100 μl) was incubated alone or with 10 μl of rat-serum-opsonised-*Escherischia coli* for ten minutes. Then 10 μl of DHR 123 was added and incubation continued for a further ten minutes. The analysis of respiratory burst activity was performed on a Facscan (fluorescence-activated cell sorting) cytofluorometer (Beckton-Dickinson) detecting mean channel fluorescence (mcf). A minimum of 5000 cells was collected and analysed using Lysis II software (San Jose, California).

Statistical analysis. Data from both sections of the study were expressed as the mean ±SEM. The results were analysed using analysis of variance for the comparison of multiple means (ANOVA) with *post-hoc* Scheffe test analysis. Significance was set at $p < 0.05$.

Results

Experiment 1

Effect of ischaemia-reperfusion on muscle function. Muscle contractile function was expressed as the peak tension (in grams of 'force') achieved by each muscle for both twitch and tetanic contractions. IRI impaired muscle twitch contraction (47 ± 5.9 g) compared with the control group (160.7 ± 23.1 g; $p < 0.01$). Pretreatment with vitamin C preserved twitch function after IRI (74.7 ± 4.6; $p < 0.05$ *v* IRI) (Fig. 1). Muscle tetanic contraction was also significantly blunted by IRI (239.4 ± 18.7) compared with the control group (451.6 ± 47.7; $p < 0.01$). Supplementation with vitamin C ameliorated the IRI-induced fall in tetanic contraction (394.7 ± 28.7; $p < 0.05$ *v* IRI) (Fig. 2).

Effect of ischaemia-reperfusion on muscle neutrophil infiltration. IRI resulted in a considerable increase in

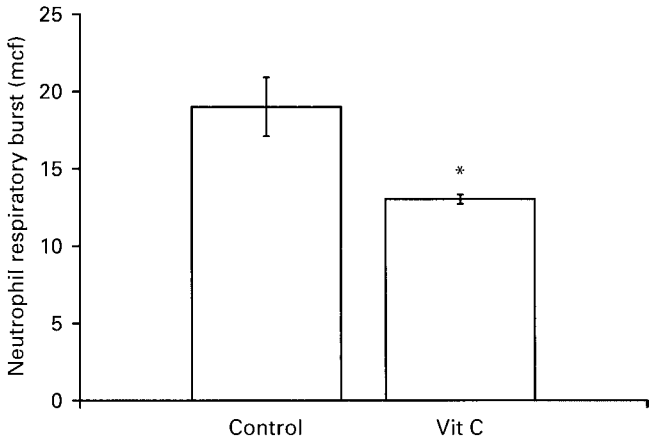

Fig. 5

Effect of IRI on the mean (±SEM) neutrophil respiratory burst activity (*$p < 0.01$ *v* control (ANOVA)).

muscle neutrophil infiltration as indicated by a rise in MPO activity from 5.38 ± 0.6 units/g in the control group to 8.21 ± 0.65 units/g in the IRI group ($p < 0.05$). The increase was reduced by pretreatment with oral vitamin C (5.14 ± 1.03 units/g; $p < 0.05$) (Fig. 3).

Wet-to-dry ratio. IRI produced a rise in tissue oedema as assessed by the wet-to-dry ratio when compared with control muscle (1.28 ± 0.6 g *v* 0.93 ± 0.09 g; $p < 0.01$). Administration of vitamin C before injury led to a significant reduction in tissue oedema (1.01 ± 0.04; $p < 0.05$ *v* IRI) (Fig. 4).

Experiment 2

Neutrophil respiratory burst activity. Pretreatment with vitamin C reduced neutrophil respiratory burst activity significantly (13.02 ± 0.3 mcf) compared with the untreated control group (19.01 ± 1.9 mcf; $p < 0.01$) (Fig. 5).

Discussion

IRI is commonly encountered in acute surgical and medical settings, and causes muscle necrosis in compartment syndrome, thromboembolism of the lower limb or coronary arteries and pneumatic tourniquet compression. While considerable advances have been made in reducing ischaemia times by revascularisation procedures, pharmacological modification of the inflammatory cascade activated by reperfusion may now be possible.

Neutrophil aggregation is the most typical finding related to IRI in many tissue models,[2,3] and the immunological steps responsible for this are becoming apparent. In IRI, the release of activated oxygen species (H_2O_2) by hypoxic/anoxic endothelium appears to be a primary event in the initiation of local and systemic inflammation.[22] T-lymphocytes release tumour necrosis factor-α (TNF-α) in response to hypoxic endothelial H_2O_2 which in turn upregulates endothelial E-selectin.[23,24] Kokura et al[24] recently reported that this TNF-α release and E-selectin expression is governed by interactions between adhesion molecules on T lymphocytes (very late antigen-4) and endothelium (vascular cell adhesion molecule-1).[24] Neutrophil adhesion in IRI is determined to a large extent by levels of selectins on damaged endothelium.[25-27] Adherent neutrophils subsequently migrate across the endothelium into the underlying ischaemic tissue and release activated oxygen species via MPO. The entire process of neutrophil chemoattraction, adhesion and activation prolongs cellular injury long after tissue ischaemia has been reversed.

There are a number of putative interventions to retard IRI. Immunological blockade of adhesion molecules and cytokines slows recruitment of neutrophils while their removal/inactivation is known to ameliorate IRI.[3,4,7,8] Antioxidants such as taurine, vitamin C and n-acetyl cysteine have been used to scavenge MPO-derived activated oxygen species.[9,10,28] Superoxide dismutase also preserves muscle function after IRI by way of a similar scavenging mechanism.[29] Our study has shown that ischaemia and reperfusion of the cremaster muscle results in significant microvascular injury characterised by the infiltration of neurophils (increased MPO activity) and tissue oedema (increased wet-to-dry ratio). IRI-induced muscle injury is reflected functionally by reduced muscle twitch and tetanic contraction forces. Oral pretreatment with vitamin C significantly reduced the functional, immunological (MPO) and microvascular (oedema) effects of IRI in this model. These results are in agreement with other groups who have examined the effects of antioxidants in skeletal muscle and myocardial models.[30,31] Hirose et al[32] and Hirose, Yamaga and Takagi[33] found that intravenous administration of a vitamin E/vitamin C diester reduced the acute tissue oedema, the functional status and the histological and biochemical markers of damage to the skeletal muscle in the rat. No study to date, however, has assessed the effect of oral presupplementation with vitamin C on muscle damage after IRI.

Vitamin C rapidly reaches a steady state in human plasma at doses greater than 200 mg per day.[15] Neutrophils, however, saturate at doses greater than 100 mg/day and accumulate vitamin C preferentially, achieving concentrations 14 times greater than plasma.[15] This preferential accumulation may explain its particular utility in moderating neutrophil-mediated IRI as seen in our study. Vitamin C acts by scavenging reactive oxygen species via rapid aqueous-phase electron transfer, thereby reducing adhesion of neutrophils to endothelium.[11] It decreases both the generation of oxygen free radicals by neutrophils and subsequent lipid peroxidation.[9,34,35] The systemic nature of the activity of vitamin C is shown by the reduction in circulating neutrophil respiratory burst after five days of oral treatment. By lowering the proinflammatory activity of neutrophils distant to the site of injury, pretreatment with vitamin C may decrease subsequent tissue damage when these neutrophils are chemoattracted to the site of IRI.

The results may be applicable to clinical practice, espe-

cially elective orthopaedic surgery. The neutrophil-based effects of the high dosage of vitamin C required in the rat model can be achieved at a relatively low dose in man (400 mg per day),[15] and the short period of pretreatment required could reduce potential side-effects.[12] Preadministration of oral vitamin C to patients undergoing operations which require prolonged tourniquet times may reduce the severity of potential IRI of the skeletal muscle distal to the pneumatic cuff. Also, reducing the generation of oxygen free radicals in neutrophils could have the converse benefit of allowing longer IRI-free tourniquet times. Furthermore, the systemic effects of IRI such as pulmonary oedema and cardiac dysfunction may also be avoided.

Our study has shown therefore that short-term oral therapy with vitamin C attenuates reperfusion-induced injury in skeletal muscle by the antioxidant effects on neutrophils. We suggest that clinical trials of short-term treatment with oral vitamin C may be useful to investigate possible benefits in reducing IRI in orthopaedic patients undergoing surgery with prolonged tourniquet times.

This study was supported by a research grant from the Cappagh Hospital Trust, Finglas Dublin 11, Ireland.

No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.

References

1. **Welbourn CR, Goldman G, Paterson IS, et al.** Pathophysiology of ischaemia reperfusion injury: central role of the neutrophil. *Br J Surg* 1991;78:651-5.
2. **Milazzo VJ, Ferrante RJ, Sabido F, et al.** Time course of leukocyte adhesion to endothelium in ischemia-reperfusion. *J Surg Res* 1996;61:139-42.
3. **Crinnion JN, Homer-Vanniasinkam S, Parkin SM, Gough MJ.** Role of neutrophil-endothelial adhesion in skeletal muscle reperfusion injury. *Br J Surg* 1996;83:251-4.
4. **Weiser MR, Gibbs SA, Valeri CR, Shepro D, Hechtman HB.** Anti-selectin therapy modifies skeletal muscle ischemia and reperfusion injury. *Shock* 1996;5:402-7.
5. **Romson JL, Hook BG, Kunkel SL, et al.** Reduction of the extent of ischemic myocardial injury by neutrophil depletion in the dog. *Circulation* 1983;67:1016-23.
6. **Engler RL, Dahlgren MD, Morris DD, et al.** Role of leukocytes in response to acute myocardial ischemia and reflow in dogs. *Am J Physiol* 1986;251:314-23.
7. **Sadasivan KK, Carden DL, Moore MB, Korthuis RJ.** Neutrophil mediated microvascular injury in acute, experimental compartment syndrome. *Clin Orthop* 1997;339:206-15.
8. **Ferrante RJ, Hobson RW, Miyasaka M, Granger DN, Duran WN.** Inhibition of white blood cell adhesion at reperfusion decreases tissue damage in postischemic striated muscle. *J Vasc Surg* 1996;24:187-93.
9. **Kearns SR, Kelly CJ, Barry M, et al.** Vitamin C reduces ischaemia-reperfusion-induced acute lung injury. *Eur J Vasc Endovasc Surg* 1999;17:533-6.
10. **Barry MC, Kelly CJ, Abdih H, et al.** Differential effects of lower limb revascularisation on organ injury and the role of the amino acid taurine. *Eur J Vasc Endovasc Surg* 1997;13:193-201.
11. **Lehr HA, Frei B, Olofsson AM, Carew TE, Arfors KE.** Protection from oxidized LDL-induced leukocyte adhesion to microvascular and macrovascular endothelium in vivo by vitamin C but not by vitamin E. *Circulation* 1995;91:1525-32.
12. **Podmore ID, Griffiths HR, Herbert KE, et al.** Vitamin C exhibits pro-oxidant properties. *Nature* 1998;392:559.
13. **Mirvish SS.** Effects of vitamins C and E on N-nitroso compound formation, carcinogenesis and cancer. *Cancer* 1986;58 (8 Suppl):1842-50.
14. **Bendich A, Langseth L.** The health effects of vitamin C supplementation: a review. *J Am Coll Nutr* 1995;14:124-36.
15. **Levine M, Conry-Cantilena C, Wang Y, et al.** Vitamin C pharmacokinetics in healthy volunteers: evidence for a recommended dietary allowance. *Proc Natl Acad Sci USA* 1996;93:3704-9.
16. **Baez S.** An open cremaster muscle preparation for the study of blood vessels by in vivo microscopy. *Microvasc Res* 1973;5:384-94.
17. **Taylor RG, Fowler WM.** Fast and slow skeletal muscles: simultaneous in vitro study. *Arch Phys Med Rehabil* 1976;57:223-8.
18. **Hill DK.** Resting tension and the form of the twitch of rat skeletal muscle at low temperature. *J Physiol* 1972;221:161-71.
19. **Close R, Hoh JF.** The after-effects of repetitive stimulation on the isometric twitch contraction of rat fast skeletal muscle. *J Physiol* 1968;197:461-77.
20. **Laight DW, Lad N, Woodward B, Waterfall JF.** Assessment of myeloperoxidase activity in renal tissue after ischemia/reperfusion. *Eur J Pharmacol* 1994;292:81-8.
21. **Rothe G, Oser A, Valet G.** Dihydrorhodamine 123: a new flow cytometric indicator for respiratory burst activity in neutrophil granulocytes. *Naturwissenschaften* 1988;75:354-5.
22. **Kokura S, Wolf RE, Yoshikawa T, Granger DN, Aw TY.** T-lymphocyte-derived tumor necrosis factor exacerbates anoxia-reoxygenation-induced neutrophil-endothelial cell adhesion. *Circ Res* 2000;86:205-13.
23. **Gaines GC, Welborn MB, Moldawer LL, et al.** Attenuation of skeletal muscle ischemia/reperfusion injury by inhibition of tumor necrosis factor. *J Vasc Surg* 1999;29:370-6.
24. **Kokura S, Wolf RE, Yoshikawa T, Granger N, Aw TY.** Postanoxic T lymphocyte-endothelial cell interactions induce tumour necrosis factor-alpha production and neutrophil adhesion: role of very late antigen-4/vascular cell adhesion molecule-1. *Circ Res* 2000;86:1237-44.
25. **Weyrich AS, Ma XY, Lefer DJ, Albertine KH, Lefer AM.** In vivo neutralization of P-selectin protects feline heart and endothelium in myocardial ischemia and reperfusion injury. *J Clin Invest* 1993;91:2620-9.
26. **Ma XL, Weyrich AS, Lefer DJ, et al.** Monoclonal antibody to L-selectin attenuates neutrophil accumulation and protects ischemic reperfused cat myocardium. *Circulation* 1993;88:649-58.
27. **Kanwar S, Smith CW, Kubes P.** An absolute requirement for P-selectin in ischemia/reperfusion-induced leukocyte recruitment in cremaster muscle. *Microcirculation* 1998;5:281-7.
28. **Davreux CJ, Soric I, Nathens AB, et al.** N-acetyl cysteine attenuates acute lung injury in the rat. *Shock* 1997;8:432-8.
29. **Bowler DJ, McLaughlin R, Kelly CJ, et al.** Recombinant human manganese superoxide dismutase attenuates early but not delayed skeletal muscle dysfunction following reperfusion injury. *Eur J Vasc Endovasc Surg* 1999;18:216-21.
30. **Bushell A, Klenerman L, Davies H, Grierson I, Jackson MJ.** Ischemia-reperfusion-induced muscle damage: protective effect of corticosteroids and antioxidants in rabbits. *Acta Orthop Scand* 1996;67:393-8.
31. **Kuzuya T, Hoshida S, Nishida M, et al.** Role of free radicals and neutrophils in canine myocardial reperfusion injury: myocardial salvage by a novel free radical scavenger, 2-octadecylascorbic acid. *Cardiovasc Res* 1989;23:323-30.
32. **Hirose J, Yamaga M, Ide J, Tanoue M, Takagi K.** Reduced ischemia-reperfusion injury in muscle: experiments in rats with EPC-K1, a new radical scavenger. *Acta Orthop Scand* 1997;68:369-73.
33. **Hirose J, Yamaga M, Takagi K.** Reduced reperfusion injury in muscle: a comparison of the timing of EPC-K1 administration in rats. *Acta Orthop Scand* 1999;70:207-11.
34. **Herbaczynska-Cedro K, Wartanowicz M, Panczenko-Kresowska B, et al.** Inhibitory effect of vitamins C and E on the oxygen free radical production in human polymorphonuclear leucocytes. *Eur J Clin Invest* 1994;24:316-9.
35. **Wen Y, Cooke T, Feely J.** The effect of pharmacological supplementation with vitamin C on low-density lipoprotein oxidation. *Br J Clin Pharmacol* 1997;44:94-7.