Mononuclear Cells From Dogs With Acute Lung Allograft Rejection Cause Contraction of Pulmonary Arteries

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Purpose Experiments were designed to determine whether or not leukocytes activated by acute pulmonary rejection cause contractions of isolated pulmonary arteries.

Methods and Results Separate suspensions of (a) polymorphonuclear cells (>95%) and (b) mononuclear cells (85% lymphocytes/10% monocytes/5% polymorphonuclear cells), respectively, were obtained from the arterial blood of four groups of adult male mongrel dogs: unoperated dogs (controls), dogs with single-lung autotransplants, dogs with rejecting single-lung allotransplants, and unoperated dogs treated with the same immunosuppressants as allotransplanted dogs. These suspensions were added to rings of control intralobar pulmonary arteries suspended in organ chambers for measurement of isometric force. The endothelium was removed mechanically from selected rings. No significant change in basal tension of pulmonary arterial rings occurred by adding suspensions of polymorphonuclear cells from any of the four groups of dogs. Significant cell-number-dependent increases in tension occurred with suspensions of mononuclear cells from unoperated dogs, autotransplanted dogs, and unoperated, medicated dogs. These increases in tension were less in rings with compared to those without endothelium. Addition of a synthetic analogue of l-arginine abolished this difference. Suspensions of mononuclear cells from rejecting allotransplanted dogs caused significantly greater contractions in rings with endothelium than those observed with suspended cells from either unoperated, autotransplanted dogs or unoperated, medicated dogs. Addition of superoxide dismutase plus catalase or an antagonist of endothelin-A receptors (BQ-123) reduced contractions in rings with endothelium but not in those without endothelium to suspensions of mononuclear cells from rejecting allotransplanted dogs.

Conclusions The results of this study suggest that mononuclear cells cause contraction of pulmonary arteries, which can be partially inhibited by endothelium-derived nitric oxide. However, if the mononuclear cells are activated by acute pulmonary rejection, contractions are no longer inhibited by the endothelium. Under conditions of rejection, contractions are mediated in part by oxygen radicals and endothelin(s).

Key Words • endothelin • free radicals • nitric oxide • transplantation

Lung transplantation is an accepted therapy for certain patients with end-stage pulmonary disease. However, episodes of acute allograft rejection represent an important clinical problem. The strategic position of the vascular endothelium between the circulating blood of the recipient and the transplanted organ suggest its potential involvement in allograft rejection. Endothelial cells act as antigen presenting cells,1 and leukocyte-endothelial adhesion is necessary for transendothelial migration of immunologically active cells and cytotoxic activity associated with rejection.2-5 In addition, endothelial cells produce factors such as nitric oxide, endothelin, and oxygen-derived free radicals, which affect the tone of the underlying smooth muscle, cell proliferation, and leukocyte adhesion.6-14 Therefore, endothelial cells may participate in regulating not only antigenicity of transplanted organs but also vascular resistance and obliterator processes associated with transplantation.

During episodes of acute rejection, expression of endothelium-dependent relaxations to agonists like adenosine diphosphate are reduced in pulmonary arteries of allotransplanted lung.15 One interpretation of these results is that during rejection the ability of the endothelial cells to produce vasodilatory substances is reduced. Little is known about how rejection-activated leukocytes affect vascular and, in particular, endothelial function. Therefore, experiments were designed to define responses of pulmonary arteries to rejection-activated leukocytes and to identify possible mediators of those responses.

Methods

Four groups of adult male mongrel dogs (21 to 25 kg) were studied: unoperated dogs, dogs with single-lung autotransplants, dogs with single-lung allotransplants, and unoperated dogs medicated the same way as were the allotransplanted dogs.

Operative Procedure

Surgical techniques, anesthesia, and analgesia for single-lung autotransplantation and allotransplantation have been reported previously.15 There were no significant differences in ischemic times within or between autotransplanted and allotransplanted groups (mean 60±10 minutes).

Postoperative Care

All operated animals received intravenous heparin (1000 U) every 4 hours for the first 12 hours. Allotransplanted animals
were immunosuppressed with cyclosporin (initially 4 mg/kg per day) adjusted to maintain plasma concentrations between 200 and 300 mg/mL, azathioprine (2 mg/kg per day), and methylprednisolone acetate (125 mg at the time of reperfusion followed by 0.3 mg/kg per day). All autotransplanted and allotransplanted dogs received prophylactic antibiotics (gentamicin 80 mg/day, clindamycin 600 mg/day).

On postoperative day 5 each allotransplanted animal had a chest radiograph. If the lung field was clear, an open-lung biopsy was taken under general anaesthesia to establish the presence or absence of rejection, after which immunosuppression was discontinued. Three days later another chest radiograph was obtained. If the allotransplanted lung field was opacified, the dog was used for an experiment. Allotransplanted dogs were killed on postoperative days 8 to 10 (ie, after 3 to 5 days without immunosuppression); all autotransplanted dogs were killed on postoperative day 8. Coronary and renal arteries from transplanted dogs were used by other investigators in unrelated studies. Unoperated, medicated dogs were maintained on the same immunosuppressants and antibiotics as the allotransplanted dogs. Immunosuppressants were discontinued after 5 days and the animals were studied 3 days later. For euthanasia, all animals were anesthetized (30 mg/kg pentobarbital IV) and exsanguinated.

**Histopathology**

The upper lobes of all transplanted and unoperated (native) lungs were inflated with 10% buffered formalin and fixed for at least 24 hours before histological sectioning and staining with hematoxylin and eosin. The severity of rejection was scored from grade 0 (no rejection) to grade 4 (severe rejection) according to the International Society for Heart Transplantation Working Formulation by an experienced pathologist blinded to the origin of the tissue.

**Bacteriology**

All animals were screened for infection and parasitic infestation before the study. Bacterial and fungal cultures were performed on tracheal mucus of the transplanted lungs and peripheral venous blood at the time of euthanasia; animals with confirmed infection were excluded.

**Isolation of Leukocytes**

Heparinized (10 U/mL) arterial blood (240 mL) was obtained from unoperated, autotransplanted, and allotransplanted dogs and unoperated, medicated dogs on the day they were killed.

The blood was aliquoted into 30-mL units, which were mixed with 12 mL of EPS solution (0.3% trisodium EDTA, 0.9% sodium chloride NaCl, 0.03% potassium phosphate K2HPO4, pH 7.0), 4 mL of 5% trisodium EDTA, and 4 mL of 2% methyl cellulose solution. The tubes remained undisturbed at an angle of 45° for 25 minutes after which the leukocyte rich plasma was drawn off.

**Preparation of Ficoll-Hypaque Two-Step Density Gradients**

Two different densities of Ficoll were required for this procedure. Ficoll-Paque (Ficoll 400, 5.7 g; diatrizoate sodium edate calcium disodium, 9 g per 100 mL, Pharmacia LKB, Biotechnology AB) has a specific gravity (sg) of 1.077. The addition of 34% by volume of 50% Hypaque solution raised the specific gravity to 1.150. Ficoll-Paque (10 mL, 1.077 sg) was layered onto 10 mL of Ficoll-Hypaque (1.150 sg), creating an interface between the two densities. Leukocyte-rich plasma (30 mL) obtained after erythrocyte sedimentation was then layered onto this two-step density gradient, and the tubes were centrifuged for 25 minutes at 1800 rpm.

Mononuclear cells (MNCs) formed a layer on top of the Ficoll-Paque, while polymorphonuclear leukocytes (PMNs) passed through this layer, forming a layer on top of the more-dense Ficoll-Hypaque. Platelet-rich plasma overlying the MNCs was discarded, and the MNCs were harvested. The Ficoll-Paque layer over the PMNs was removed, enabling harvest of the PMNs. The MNCs were washed twice in Krebs-Ringer bicarbonate solution (control solution [mmol/L]; NaCl 118.3, KCl 4.7, CaCl2 2.5, MgSO4 1.2, K2HPO4 1.2, NaHCO3 25.0, calcium disodium EDTA 0.25, and glucose 11.1). PMNs also were washed twice after residual erythrocytes had been lysed with ACK buffer (NH4Cl 0.1 mol/L).

After MNCs were washed, they were resuspended in control solution at an approximate concentration of 106 cells/mL. This suspension was incubated in plastic Petri dishes at 37°C in a humidified 10% CO2 atmosphere for 1 hour. This method causes phagocytic monocytes (macrophages) to become adherent to the plastic surface, leaving nonadherent lymphocytes in suspension. After 1 hour, each dish was gently swirled to dislodge any nonadherent cells, and the contents were transferred to a new dish and returned to the incubator for another hour. Nonadherent cells were harvested, washed, and resuspended in control solution. A sample was stained with crystal violet to obtain a total and differential cell count with a manual hemocytometer; total counts were also obtained from a Coulter counter. Cell viability was assessed by trypan blue dye exclusion. This procedure produced cell suspensions consisting of >85% mixed lymphocytes, 10% monocytes, and 5% PMNs; viability was consistently >95%. A stock suspension of cells in control solution was made at a concentration of 3.5 × 106/mL.

After erythrocytes were lysed and washed, PMNs were resuspended in control solution before being counted and assessed in the same way as the MNCs. Suspensions of PMNs contained >95% PMNs with >95% viable cells. A stock cell suspension of 3.5 × 106/mL was made.

**Organ Chamber Experiments**

Intralobar pulmonary arteries were dissected from the lungs of unoperated dogs and carefully cleaned of connective tissue! These vessels were cut into rings 4 to 5 mm long, and in randomly selected rings, endothelium was removed mechanically by gently rubbing the luminal surface with the tip of a pair of watchmakers’ forceps. All rings were suspended between a fixed point and a force transducer (Grass FT3) for the measurement of isometric force by two stainless steel wires inserted into the lumen of the ring. Individual rings then were placed into 3.5-mL organ chambers filled with Krebs-Ringer bicarbonate solution (control solution) maintained at 37°C and bubbled with a 95% O2/5% CO2 mixture. The rings were allowed to equilibrate for a passive tension of less than 1 g for 30 minutes. After this, each ring was progressively stretched to the optimal point on its length-tension curve as determined by the tension developed to potassium chloride (KCl; 20 mmol/L) at each level of stretch (1-g increments). The rings were allowed to equilibrate at the optimal tension (basal tension) for 30 minutes. A maximal contraction to KCl (60 mmol/L) was obtained, following which the integrity of the endothelium was determined by the presence of a relaxation to acetylcholine (3 × 10−7 mol/L) in rings with but not without endothelium. The chambers then were washed with control solution and equilibrated for a further 30 minutes until the tension had returned to basal levels. Lymphocyte-enriched MNCs or PMNs (5 × 106 to 107 per mL of organ chamber fluid) from either unoperated dogs, single-lung autotransplanted dogs, single-lung allotransplanted dogs with acute rejection, or unoperated, medicated dogs were added to pairs of rings (ie, with and without endothelium) and cumulative cell number concentration-response curves were recorded. Responses to lymphocyte-enriched MNCs or PMNs (250 × 106/mL) from autotransplanted and allotransplanted animals were obtained in the absence and presence of either superoxide dismutase (150 U/mL) plus catalase (1200 U/mL), or the endothelin-A (ET-A) receptor antagonist cyclo[D-Trp-D-Asp-Pro-D-Val-Leu] (BQ-123, 10−6 mol/L).
mol/L), or BQ-123 (10^{-4} \text{ mol/L}) plus N^{0}-monomethyl-L-arginine (L-NMMA, 10^{-6} \text{ mol/L}), a synthetic analog of L-arginine. The duration of organ chamber experiments from the removal of tissue to completion of the cell number concentration-response curve was 6 hours. The cell number concentration-response curve took about 50 minutes to complete.

**Endothelin-1 (ET-1) Radioimmunoassay**

Cell-free supernatants of PMNs, lymphocytes, and cell-free fluid from the organ chamber were analysed for ET-1 by a radioimmunoassay extraction method (Amersham International). The fluid from the organ chamber was removed at the end of the experiments after the cells had been added. It then was centrifuged at 1800 rpm for 10 minutes. The cell-free supernatant was frozen at -70°C until the assay was performed. The lower sensitivity of the assay was 0.5 pg/mL. The assay cross-reacts with ET-1 (100%), endothelin-2 (204%), and endothelin (38%), and yields 79±9% recovery.17

**Scanning Electron Microscopy**

Pulmonary arterial rings with endothelium that had been exposed to lymphocyte-enriched suspensions in the organ chambers were opened longitudinally and pinned out with the endothelium exposed. They were fixed with Trump’s solution (1% glutaraldehyde/4% formaldehyde) before being dehydrated in graded alcohols. Specimens then were critical-point dried before being coated with gold and palladium by vacuum evaporation (Denton Vacuum Evaporator/DV-502A). Specimens then were examined with a scanning electron microscope (JEOL 6400 SEM).

**Statistical Analysis**

The results are expressed as mean±SEM. In all experiments, n equals the number of animals from which cells were isolated. The responses of rings to the addition of cells are expressed as a percentage of the contraction caused by 60 mmol/L KCl for each ring (percent of maximal contraction to KCl). The area under each cell-response curve was calculated and used to determine differences within groups. Student’s t test for paired observations was used within each group (ie, to compare responses of rings with and without endothelium from the same dog or to determine the effects of inhibitors). One-way ANOVA was used to identify differences among groups. If a significant F was found, a post hoc Scheffé's test was used to identify differences among means. A difference was considered to be significantly different when P<.05.

**Results**

All pulmonary arterial rings used in these experiments were from unoperated dogs; leukocytes were derived from either unoperated, autotransplanted, allotransplanted, or unoperated, medicated dogs.

**PMNs**

No significant change occurred in basal tension of pulmonary arterial rings with and without endothelium in response to PMNs (5×10^4 to 3×10^6 cells per mL) from unoperated, autotransplanted, allotransplanted, rejecting dogs (Fig 1).

**Lymphocyte-Enriched MNCs**

Lymphocyte-enriched MNCs (lymphocytes; 5×10^4 to 3×10^6 per mL) caused cell number-dependent contractions of rings of pulmonary arteries with and without endothelium. The magnitude of contraction depended upon the source of the lymphocytes (Fig 1).

**Unoperated Dogs**

Lymphocytes from unoperated dogs caused cell number-dependent contractions that were significantly greater in rings of pulmonary arteries without endothelium compared to with endothelium (Fig 2). The maximal contractions (at lymphocyte concentration of 5×10^6/mL) were 34.6±7.5% of a contraction caused by 60 mmol/L of KCl in rings with endothelium, and 54.4±7.4% in rings without endothelium.

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**Fig 1.** Tracing of response of canine pulmonary arteries with endothelium to leukocytes from unoperated dogs or dogs with rejecting lung allografts.

**Fig 2.** Cumulative cell-response curves to lymphocytes from unoperated dogs (left panel), single-lung autotransplanted dogs (middle panel), and allotransplanted dogs with acute rejection (right panel) on pulmonary arterial rings from unoperated dogs. Results are expressed as a percentage of the maximal contraction to KCl (60 mmol/L). Contraction to KCl of rings with and without endothelium, respectively, averaged (mean±SEM; grams) for unoperated group, 5.2±0.6 and 4.7±0.7, n=6; autotransplanted group, 4.9±0.8 and 4.9±0.5, n=3; for allotransplanted group, 5.2±0.4 and 6.1±0.6, n=12. Asterisks denote statistically significant difference in maximal tension between rings with and without endothelium (Student’s t test for paired observations, P<.05).
Autotransplanted Dogs

Lymphocytes from autotransplanted dogs also caused cell-dependent contractions of pulmonary arteries, which were significantly greater in rings without compared to with endothelium (Fig 2). The maximal contractions of rings with or without endothelium were not statistically different from those caused by cells from unoperated dogs.

Allotransplanted Dogs

Lymphocytes from allotransplanted dogs with acute rejection caused comparable contractions in rings of pulmonary artery with and without endothelium (Fig 2). Contractions of rings with but not without endothelium were significantly greater than those seen in response to lymphocytes from unoperated, autotransplanted, or unoperated, medicated dogs.

Unoperated, Medicated Dogs

Lymphocytes from unoperated dogs medicated in the same way as were the allotransplanted animals caused cell number–dependent increases in tension of pulmonary arteries. The contractions were significantly greater in rings without compared to with endothelium. In response to 500×10^9/mL cells, the tensions were 14.1±5.2% and 59.7±7.1% of the tensions to 60 mmol/L KCl (3.8±0.7 g and 5.1±0.4 g; n=4) in rings with and without endothelium, respectively. The contractions of rings with endothelium to lymphocytes from unoperated, medicated dogs were significantly less than those from allotransplanted, rejecting dogs.

Superoxide Dismutase Plus Catalase

Superoxide dismutase (150 U/mL) plus catalase (1200 U/mL) had no effect on contractions of rings with endothelium to lymphocytes from autotransplanted dogs (Figs 2 and 3). However, contractions of rings with endothelium from allotransplanted rejecting dogs (Fig 2) were reduced significantly by these radical scavengers (Fig 3). In the presence of superoxide dismutase plus catalase, contractions of rings with endothelium to lymphocytes from autotransplanted and allotransplanted dogs were the same (Fig 3). Maximal contractions of rings without endothelium to cells from autotransplanted and allotransplanted animals also were comparable in the presence of superoxide dismutase plus catalase.

Endothelin Receptor A Antagonist BQ-123

The antagonist of ET-A receptors BQ-123 (10^-6 mol/L) had no effect on contractions of rings with or without endothelium to lymphocytes from autotransplanted dogs. Contractions of rings of pulmonary artery with endothelium to lymphocytes from allotransplanted rejecting dogs were reduced significantly (55%) by BQ-123 (10^-6 mol/L); there was no significant reduction in contractions of rings without endothelium (Fig 4).

BQ-123 Plus L-NMMA

While BQ-123 (10^-6 mol/L) alone did not affect contractions in response to lymphocytes from autotransplanted dogs, the combination of BQ-123 plus L-NMMA (10^-4 mol/L) significantly increased contractions of rings with endothelium (Fig 4). In the presence of the inhibitors, there was no longer a difference in contractions between rings with and without endothelium. Contractions of rings with endothelium in response to lymphocytes from rejecting dogs also were increased significantly (Fig 4). However, in the presence of both inhibitors, contraction of rings without endothelium was still significantly greater than rings with endothelium.
ET-1 Assay

ET-1 was detectable in the cell-free supernatant of suspensions of lymphocytes and PMNs and also in cell-free fluid from organ chambers. The concentration of ET-1 was 2.6±0.1 pg/mL (10⁻⁹ mol/L) from autotransplanted and allotransplanted lymphocytes at a concentration of 3.5×10⁶ cells/mL. The concentration of ET-1 was comparable in all chambers irrespective of whether they contained rings with or without endothelium or whether superoxide dismutase plus catalase, BQ-123, or L-NAME were present. The cell-free supernatant from chambers that had received 5×10⁷/mL PMNs contained a similar concentration of ET-1 compared with fluid from chambers that had received 3.5×10⁶ lymphocytes. That is, about 10 times fewer lymphocytes produced a similar amount of ET-1 when compared with PMNs.

Histological Grade of Rejection

Two allotransplanted dogs had grade 1 rejection (minimal acute rejection—scattered perivascular MNC infiltrates); the other allotransplanted animals had grade 3 rejection (moderate acute rejection—wide-spread perivascular cuffing by MNCs with extension into alveolar septae).

Scanning Electron Microscopy

Endothelial cells were present on rings after exposure to lymphocytes from unoperated or autotransplanted dogs, and few adherent lymphocytes were observed (Fig 5). After exposure to lymphocytes from dogs with rejecting allografts, a greater number of cells were adherent and were associated with endothelial cells with contracted cytoplasm exposing the underlying smooth muscle.

Discussion

The observation that peripheral blood leukocytes initiate changes in vascular tone is not new. Indeed, unstimulated and stimulated PMNs have been shown to produce activated oxygen species, nitric oxide, and endothelin.18-22 The results of the present study extend these previous observations to show that lymphocytes from circulating blood cause contraction of isolated pulmonary arteries. Further, lymphocytes that have been activated during acute allograft rejection reduce the ability of vascular endothelial cells to inhibit direct effects of the cells on the smooth muscle. The loss of buffering capacity of the endothelium may not represent denudation of the endothelium because endothelial cells were present in rings exposed to lymphocytes as determined by scanning electron microscopy. The loss of buffering capacity of the endothelium may represent soluble factors released from the lymphocytes, because contractions of rings with endothelium to lymphocytes from rejecting dogs could be manipulated pharmacologically. It is unlikely that the effects of rejection-activated cells are a result of residual effects of immunosuppressive drugs.23-25 Contractions of rings with endothelium to cells from animals treated with immunosuppressive therapy alone were less than those without endothelium. This pattern of response was similar to cells from animals not receiving immunosuppressive drugs (ie, control and autotransplant).

The vascular responses to rejection-activated lymphocytes appear to be mediated by at least three groups of endothelium- and/or leukocyte-derived factors: superoxide radicals, nitric oxide, and ET-1. This conclusion is supported by several observations. The free radical scavengers superoxide dismutase plus catalase reduced contractions of rings with endothelium to lymphocytes from dogs with rejecting allografts. This suggests that lymphocytes produce oxygen-derived free radicals. The ability of oxygen radicals to cause contractions of pulmonary arteries has been shown previously.14 In addition, superoxide radicals inhibit endothelium-dependent vasodilation in response to the calcium ionophore A23187, leukotriene D₄, and acetylcholine.26,27 It is likely that free radicals inhibited release or destroyed endothelium-derived relaxing factor in response to lymphocytes. This conclusion is supported by the observation that a difference in contraction between rings with and without endothelium was observed to rejection-activated cells in the presence of the radical scavengers superoxide dismutase plus catalase.

Nitric oxide may be one endothelium-derived factor that antagonizes the effect of lymphocyte-derived factors on the smooth muscle. This conclusion is supported by the observation that, in autotransplanted animals, contractions of rings with and without endothelium from autotransplanted dogs are the same in the presence of the inhibitor of nitric oxide, L-NAME. Factors other than nitric oxide may contribute to the endothelium-dependent inhibition of lymphocyte-mediated contraction, however. Although L-NAME increased contractions to rejection-activated cells of rings with endothelium, the difference between contractions of rings with and without endothelium was not eliminated.

ET-1 was detected in both the cell-free supernatant of lymphocytes and in fluid from organ chambers. How ET-1 participates in the responses to lymphocytes is unclear. ET-1, which contracts vascular smooth muscle, is produced by endothelial cells,28 macrophages, and PMNs.22,29 Since comparable amounts of ET-1 were detected in organ chambers containing blood vessels with and without endothelium, it is likely the peptide originated from the lymphocytes and not from the endothelium or vascular smooth muscle.

ET-1 was detected in suspensions from PMNs but at a 10-fold-lower concentration than from lymphocytes at comparable cell concentrations. This may explain why contractions in response to PMNs were not observed in the present study. There are two types of endothelin receptors on pulmonary vascular smooth muscle: types A and B. Although type A is more prevalent (60% of receptors),30 type B receptors have a higher affinity for ET-1 than ET-A receptors.31 ET-1 acting on an ET-A receptor probably does not initiate contractions of the smooth muscle (rings without endothelium) in response to the cells because contractions of the smooth muscle to lymphocytes from either autotransplanted or allotransplanted dogs were not reduced by BQ-123. ET-A receptors may be present on the smooth muscle but may have been downregulated in response to lymphocyte-derived products.

In the presence of the ET-A antagonist, the ability of the endothelium to antagonize lymphocyte-mediated contractions was greater when cells from rejecting dogs
Fig 5. Electron micrograph of the endothelial surface of rings of pulmonary arteries from unoperated dogs after exposure to lymphocytes isolated from unoperated dogs (top panel), autotransplanted dogs (middle panel), and allotransplanted rejecting dogs (bottom panel). Bar equals 10 μm, magnification ×500.
were used. These results suggest that there may be ET-A receptors on the endothelium. Activation of these receptors may inhibit production of nitric oxide, as the effect of BQ-123 was attenuated by L-NMMA. Alternatively, activation of endothelial ET-A receptors may increase production of superoxide, which in turn would inactivate endothelium-derived nitric oxide. Oxygen radicals augment the vasoconstrictor effect of ET-1. Since BQ-123 had no effect on the response to lymphocytes from autotransplanted dogs, the interaction between ET-1 and either nitric oxide or superoxide may be linked to receptor activated lymphocyte-endothelial cell interactions.

The design of the present experiments does not differentiate between effects of the various drugs on pulmonary arteries or lymphocytes. Therefore, an alternative interpretation of the data is that activation of ET-A receptors on lymphocytes stimulates production of superoxide radicals from the cells themselves. However, this process may be present only in cells activated by the rejection process, because contractions of rings with endothelium were reduced in the presence of scavengers of oxygen-derived free radicals (Figs 2 and 3).

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