Effect of angiotensin-converting enzyme inhibitor on DNA damage and inflammatory molecules expression in rabbit aortic endothelial cells cultured in vitro in hypercholesterolemic and hyperglycemic conditions

Daniel Pomaro¹, Anita L. R. Saldanha², Francisco Fonseca³, Maria Cristina Izar³, Helena Nader⁴, Dulce Casarini³, Daniel Ribeiro⁵, Tania Leme da Rocha Martinez²* and Silvia Ihara¹

¹Department of Pathology, Universidade Federal de São Paulo, São Paulo, Brazil
²Nephrology Department, BP - A Beneficência Portuguesa de São Paulo, São Paulo, Brazil
³Department of Medicine, Universidade Federal de São Paulo, São Paulo, Brazil
⁴Department of Biochemistry, Universidade Federal de São Paulo, São Paulo, Brazil
⁵Department of Biosciences, Universidade Federal de São Paulo, São Paulo, Brazil

ARTICLE INFO

ABSTRACT

Diabetes mellitus is a major risk factor for vascular diseases such as atherosclerosis. The inhibition of the renin-angiotensin system may exert a protective effect on the development of atherosclerosis. The current study aimed to verify the action of an angiotensin-converting enzyme inhibitors (ACEI) in endothelial cells that were cultured in vitro under hypercholesterolemic and hyperglycemic conditions. Rabbit aortic endothelial cells were cultured in medium with ox-LDL (30 µg) and 22.2 mM or 5.5 mM of glucose in the presence or absence of an ACEI (1 mM quinapril). The expression profiles of the inflammatory markers intracellular adhesion molecule-1 (ICAM-1) and monocyte chemoattractant protein-1 (MCP-1) were analyzed using immunocytochemistry and quantified using image analysis software. In addition, DNA damage was analyzed using the comet assay. We observed a reduction in ICAM-1 expression in endothelial cells that were treated with ACEI and cultured in medium containing a low concentration of glucose. The expression of MCP-1 was reduced in cells that were cultured with a low concentration of glucose in an ACEI-independent manner. No differences were observed in the percentage of DNA damage among the groups. Using an in vitro model, we observed that ACEI reduced the expression of inflammatory proteins in endothelial cells, and this protective effect depended on the glycemic control.

Introduction

Endothelial dysfunction is an early step in the atherosclerosis process, preceding the morphological changes in the arterial wall and several pathological conditions that impair endothelial function⁷. The increased expression of the vascular cell adhesion molecule-1 (VCAM-1) and the intracellular adhesion molecule-1 (ICAM-1) on the endothelial surface is an early event in atherogenesis². In the intima layer, oxidized LDL increases the expression of glycoproteins such as P-E selectins, VCAM-1 and ICAM-1, that mediate the adhesion of leukocytes to endothelial cells³. Monocyte chemoattractant protein-1 (MCP-1) controls the chemotaxis of mononuclear cells and is involved in the inflammatory aspect of atherogenesis by contributing to the initiation and development of vascular lesions. In diabetes mellitus, hyperglycemia can accelerate MCP-1 production in endothelial cells through a mechanism that generates reactive oxygen species⁴.

Previous studies have suggested that DNA damage occurs in the cells within the atherosclerotic plaques and plays an important role in atherogenesis and the stability of lesions. DNA damage is associated with the development and progression of atherosclerosis⁵,⁶,⁷. DNA damage is increased in patients with
coronary artery disease, and is associated with increased aortic intima-media thickness. The level of DNA damage is correlated with the presence of atherogenic risk factors such as hypertension, diabetes mellitus and smoking.

The renin angiotensin system (RAS) has an important function in cardiovascular disease and is a complex system that can accelerate the development of atherosclerosis. Angiotensin II promotes oxidative stress in the vessel wall and leads to the inactivation of nitric oxide, causing endothelial dysfunction and the development of an atheroma. The effects of angiotensin II are mediated by the G-protein coupled receptors type 1 (AT1 receptor) and type 2 (AT2 receptor). Once angiotensin II binds to the AT1 receptor, a cascade of second messengers is activated, including the activation of phospholipase C, which increases protein synthesis, mitogenesis and hypertrophy to influence the development of atherosclerosis.

Studies with angiotensin-converting enzyme inhibitors (ACEI) have demonstrated that they are effective to normalize blood pressure and reduce the cardiovascular risk. Several experimental studies with hypercholesterolemic rabbits have shown that the use of an ACEI reduces the development of atherosclerotic lesions. The proposed mechanism for the beneficial effects of ACE (angiotensin-converting enzyme) requires cytokine activity to mediate the inflammatory response and to decrease the expression of VCAM-1. The use of quinapril in hypercholesterolemic rabbits prevents the activation of NF-kB, which is a key factor to control the expression of the chemotactic factor MCP-1.

In previous studies from our group, the protective effect of an ACE inhibitor (quinapril) was observed in hypercholesterolemic rabbits as well as in diabetic and hypercholesterolemic rabbits that were treated with ACEI. In our previous study, rabbits with high plasma glucose levels were not protected by ACEI treatment in the aorta and required management strategies to regulate diabetes and prevent the development of an atheroma. In hypercholesterolemic rabbits with controlled blood glucose levels, ACEI treatment attenuates atherosclerosis, which is indicated by significant decreases in the intima/media ratio, the intimal area and the height of the plaque. Moreover, this protection is not observed in the diabetic group with marked hyperglycemia (blood glucose ≥ 250 mg/dL) that is treated with the same drug. This protection by the ACEI has been observed in hyperglycemic rabbits with blood glucose levels that have returned to normal levels. These studies have demonstrated that the protection by the ACEI in rabbits with severe hyperglycemia can be achieved once the glucose level is normalized.

One of the proposed mechanisms that may interfere with ACEI action in the atherosclerosis process during hyperglycemic conditions is the influence of hyperglycemia on the inflammatory response and the expression of vascular adhesion molecules and chemotactic factors, which participate in the recruitment and infiltration of macrophages to the subendothelial layer.

Increased levels of angiotensin II can induce oxidative stress to promote endothelial dysfunction, hypertension and atherosclerosis. DNA damage has been observed following treatment with angiotensin II in vitro, and this effect may be due to the activation of the AT1 receptor and the subsequent release of reactive oxygen species. The treatment of cultured human umbilical vein endothelial cells with the angiotensin II receptor blocker, telmisartan, modulates the levels of VCAM-1 expression and oxidative damage by acting as a hydroxyl radical scavenger.

Although many mechanisms are involved in the development and stabilization of atherosclerotic plaques, in the current study, we examined the effects of ACEI on the genotoxicity and the expression of inflammatory molecules in hypercholesterolemia and hyperglycemia in vitro. The aim of the current study was to evaluate the effect of ACEI on DNA damage and the expression of MCP-1 and ICAM-1 in rabbit aortic endothelial cells that were cultured in vitro under hypercholesterolemic and hyperglycemic conditions.

Abbreviations

ACE: angiotensin-converting enzyme; ACEI: angiotensin-converting enzyme inhibitors; AT1: type 1 receptor; AT2: type 2 receptor; FCS: fetal calf serum; ICAM-1: intracellular adhesion molecule-1; MCP-1: monocyte chemotactic protein-1; RAECs: rabbit thoracic aorta endothelial cells; RAS: renin angiotensin system; VCAM-1: vascular cell adhesion molecule-1

Material and methods

Endothelial cells culture

An endothelial cell line that was derived from the rabbit thoracic aorta endothelial cells (RAECs) was kindly provided by Dr. Helena B. Nader from the Biochemical Department of UNIFESP. RAEC cultures were established as previously described. The cells were grown in Ham’s Nutrient Mixture F-12 (Gibco-Invitrogen, Carlsbad, CA, USA) that was supplemented with 10% fetal calf serum (FCS) (Cultilab, Campinas, SP, Brazil), streptomycin (100 mg/ml) and penicillin (100 IU/ml) (Sigma-Aldrich, Chemical Co, St Louis, MO, USA) at 37°C in a humidified atmosphere with 5% CO2. The cell suspension was grown to confluence. After three or four passages, the cells were used in experiments that were performed within 24 hours of reaching confluence. Cell release was performed using 0.25% viokase/EBSS (Earle’s Balanced Salt Solution) (Sigma-Aldrich, Chemical Co, St Louis, MO, USA) for 20 min.

Experimental groups of endothelial cell culture

RAECs were grown on 13-mm-diameter glass coverslips in 24-well plates (TPP, Trasadingen, Switzerland) and used for immunocytochemical analyses. After reaching the desired confluence, the cells were allocated into eight different groups using the list that is provided below and cultured for 24 hours without FBS at 37°C and 2.5% CO2.
Treatment groups were designed as follows:
- Group I (22.2 mM glucose + 30 µg of ox-LDL)
- Group II (22.2 mM glucose + 1 mM ACEI + 30 µg of ox-LDL)
- Group III (5.5 mM glucose + 30 µg of ox-LDL)
- Group IV (5.5 mM glucose + 1 mM ACEI + 30 µg of ox-LDL)

The RAECS were incubated for 24h with ACEI (Quinapril - Accupril®, Pfizer, Cali, Colombia) in culture medium followed by 24h of serum deprivation. To determine the cytotoxicity of the compounds on the cell viability, RAECS were stained with a 0.25% (vol/vol) of trypan blue solution and the number of the viable cells was counted. The cells were stored in the well until the time of analysis and covered with 500 mL of 0.25% fish gelatin in PBS and 0.1% sodium azide.

Determination of ACE activity

The ACE activity was measured in the Laboratory of Kidney and Hormones in the Nephrology Department of the Universidade Federal de São Paulo. For determination of ACE activity, RAECS were cultured on p100 plates (TPP, Trasadingen, Switzerland) using the protocol described above. After incubation with the indicated treatments, the cells were collected and cell extracts were prepared adding a lysis buffer (10 mM HEPES, pH 7.9). The ACE activity was determined fluorometrically in a solution containing carbobenzoxy-phenylalanine-histidine-leucine (ZPhe-His-Leu, Sigma-Aldrich, Palo Alto, CA USA) as a synthetic substrate of ACE. Aliquots (10 µL) of the cell extract were incubated at 37°C with 200 mL of the substrate ZPhe-His-Leu for a period of 4 hours, and the reaction was stopped with 1.5 mL of NaOH 0.28 N. The fluorescence λ<sub>EX</sub>=360nm; λ<sub>EM</sub>= 500 nm) was read in spectrophurometer (Hitachi F-2000 Spectrofluorometer, Hitachi, Japan)30. The protein concentration of the cell extract was determined by the method described by Bradford31 using 200 mL of each sample, the Bradford kit (Bio-Rad, USA) and bovine serum albumin as a protein standard. ACE activity was expressed relative to the measured protein concentration of the same sample (µm/mL/mg of protein).

Ox-LDL Preparation

Blood samples were drawn under the fasting conditions (16-18 hours). The blood was collected in a tube with 1% EDTA (1 mg/mL) and immediately centrifuged (4°C, 10 min, 1,000 x g) to obtain the plasma. The following preservatives were added: aprotinin (0.1 unit/mL), benzamidine (2 mM), gentamicin (0.5%), chloramphenicol (0.25%) and phenylmethylsulfonyl fluoride (0.5 mM). The LDL was obtained by sequential ultracentrifugation using the Beckman ultracentrifuge with the Ti 50 rotor. The plasma was centrifuged at 100,000 x g for 18 h to obtain VLDL (density = 1.006 g/mL). After this period, a fraction of the infranatant was diluted to 1.063 g/mL potassium bromide (KBr) was added, and the samples were centrifuged for 20 h (100,000 x g) to obtain LDL (density 1.063 g/mL). The LDL in the supernatant was removed and dialyzed for 48 h using a phosphate-buffered solution (PBS containing 0.9% NaCl, 0.2% Na<sub>2</sub>HP04, 0.38% NaOH and 0.01% EDTA, pH 7.4). The proteins were quantified using a Pierce BCA protein assay kit (Thermo Scientific, Rockford, IL, USA) according to the manufacturer's instructions. The total cholesterol and triglyceride levels were obtained using enzymatic colorimetric methods (Liquiform, Labtest-Diagnostica, Brazil) according to the manufacturer’s instructions. Triglyceride values in the range of 160-220 mg/dL and cholesterol values in the range of 90-130 mg/dL were accepted as normal ranges. Normal samples were used as a source of plasma LDL and ox-LDL and were dialyzed in PBS for 24h at 4°C to remove EDTA. LDL was incubated with copper sulfate (20 µM CuSO<sub>4</sub>) for 14 h. After this time, the oxidation was blocked by the addition of 1 mM EDTA32.

Single cell gel (Comet) assay

DNA damage was analyzed using the alkaline comet assay following a method outlined by Sasaki et al.33 with some modifications. Briefly, 5 µL of the detached cells was added to 120 µL of 0.5% low-melting-point agarose at 37°C, layered onto a pre-coated slide with 1.5% regular agarose and covered with a coverslip. After a brief period to allow the agarose to solidify in a refrigerator, the coverslips were removed and the slides were immersed in lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris-HCl buffer, pH 10, 1% sodium sarcosinate with 1% Triton X-100 and 10% DMSO) for approximately 1 h. The slides were placed in an alkaline buffer (pH > 13) for 20 min and then electrophoresed for 20 min at 0.7 V/cm and 300 mA. After electrophoresis, the slides were neutralized in 0.4 M Tris-HCl buffer (pH 7.5), fixed in absolute ethanol and stored until analysis. The slides were stained with 40 µl EtBr (20 ug/mL) and analyzed using a fluorescence microscope. To prevent additional DNA damage, all of the steps were performed under reduced illumination. A total of 50 randomly captured comets per sample (25 cells from each slide) were examined blindly at 400x magnification. The observer used a fluorescence microscope that was connected through a black and white camera to an image analysis system (Perceptive Instruments, Suffolk, Haverhill, UK), which was calibrated according to the manufacturer’s instructions. The computerized image analysis system acquired images, computed the integrated intensity profiles for each cell, estimated the comet cell components and then evaluated the range of the derived parameters. Undamaged cells had an intact nucleus without a tail, whereas damaged cells have the appearance of a comet. To measure DNA damage, two image analysis system parameters were considered as follows: tail intensity (% migrated DNA) and tail moment (the product of the tail length and the fraction of DNA in the comet tail)34,35.

Immunocytochemistry and morphometric analysis

Immunocytochemistry was performed on coverslips plated with RAECS using monoclonal antibodies against ICAM-1 (1:200, Dako, CA, USA) and MCP-1 (1:400, Santa Cruz Biotechnology, CA, USA). CD34 (1:200, Dako, CA, USA) was used for the characterizations of endothelial cells. A three-step method was used and included the complex streptavidin-biotin (LSAB, Dako, CA, USA) and the substrate H<sub>2</sub>O<sub>2</sub> with 3,3'-diaminobenzidine (DAB) chromogen (Sigma, Germany). The
such findings are summarized in Figure 2.

Regarding hypercholesterolemic conditions, our results show no patterns for genotoxicity in any of the groups. In particular, the ACEI treatment of cells under hypercholesterolemic and hyperglycemic conditions in vitro and treated with ACEI. (I) 22.2 mM glucose + 30µg ox-LDL; (II) 22.2 mM glucose + 30µg ox-LDL + 1 mM ACEI; (III) 5.5 mM glucose + 30µg ox-LDL; (IV) 5.5 mM glucose + 30µg ox-LDL + 1 mM ACEI. p>0.05; ANOVA test.

ICAM-1 and MCP-1 expression

The immunoexpression of ICAM-1 in ACEI-treated RAECs was significantly reduced when the glucose concentration in the medium was 5.5 mM (group IV). Increasing the glucose concentration to 22.2 mM did not provide significant reductions following ACEI treatment (group II). We observed a trend showing lower values in the ACEI-treated groups that were cultured in medium with high glucose levels (group II). In addition, the presence of ox-LDL in the medium did not affect the immunoexpression of ICAM-1 in the cells (Figure 3). The immunocytochemical expression of MCP-1 was significantly reduced in all groups that were incubated with a low concentration of glucose (groups III and IV) compared to those incubated with a high glucose concentration (groups I and II). Unlike the immunoexpression of ICAM-1, the MCP-1 was significantly lower in all groups of cells in the presence of 5.5 mM glucose independent of ACEI treatment. In addition, the presence of ox-LDL did not affect MCP-1 expression (Figure 4).

Figure 1. ACE activity in the extract of RAECs cultured in vitro and separated into groups based on glucose concentration and ACEI treatment as follows: (I) 22.2 mM glucose + 30µg ox-LDL; (II) 22.2 mM glucose + 30µg ox-LDL + 1 mM ACEI; (III) 5.5 mM glucose + 30µg ox-LDL; (IV) 5.5 mM glucose + 30µg ox-LDL + 1 mM ACEI. *p<0.05; groups II, IV < I, III. ANOVA-Newman Keuls tests.

DNA damage (Comet Assay)

The results of the single cell gel (comet) assay demonstrated that treatment with high concentrations of glucose did not induce DNA damage in endothelial cells. Similarly, the ACEI-treated groups did not induce statistically significant differences compared to the other groups. Regarding hypercholesterolemic conditions, our results show no patterns for genotoxicity in any of the groups. In particular, the ACEI treatment of cells under hypercholesterolemic conditions did not promote remarkable genetic changes. Such findings are summarized in Figure 2.
in cells cultured in medium with low glucose levels independent of blood pressure elevation. These results suggest that ACEI treatment influences the expression of vascular adhesion molecules. The presence of ACEI reduced the immunoexpression of ICAM-1 and MCP-1 in the intima of aorta is significantly lower in the hypercholesterolemic and normoglycemic groups that were treated with ACEI compared to hypercholesterolemic and hyperglycemic groups (in press). These results suggest that ACEI treatment influences the expression of ICAM-1 and MCP-1 to mediate the inflammation in the early stages of atherogenesis, and the efficacy of ACEI is closely related to the glycemic effects on macrovascular disease.

Figure 3. ICAM-1 expression (pixel) determined by immunocytochemical reaction, in endothelial cells exposed to hypercholesterolemic and hyperglycemic conditions in vitro and treated with ACEI. (I) 22.2 mM glucose + 30μg ox-LDL; (II) 22.2 mM glucose + 30μg ox-LDL + 1 mM ACEI; (III) 5.5 mM glucose + 30μg ox-LDL; (IV) 5.5 mM glucose + 30μg ox-LDL + 1 mM ACEI. An effective protection was conferred by ACEI in cells cultured in medium with low glucose levels. *p<0.01; groups IV < I, III. ANOVA – Newman Keuls test.

Figure 4 - MCP-1 expression (pixel) determined by immunocytochemical reaction, in endothelial cells exposed to hypercholesterolemic and hyperglycemic conditions in vitro and treated with ACEI. (I) 22.2 mM glucose + 30μg ox-LDL; (II) 22.2 mM glucose + 30μg ox-LDL + 1 mM ACEI; (III) 5.5 mM glucose + 30μg ox-LDL; (IV) 5.5 mM glucose + 30μg ox-LDL + 1 mM ACEI. An effective protection was conferred by ACEI in cells cultured in medium with low glucose levels. Low MCP-1 expression was observed in cells that were cultured in medium containing low glucose levels independent of ACEI treatment. *p<0.05; groups III, IV < I, II. ANOVA – Newman Keuls test.

Discussion

We observed in the current study that ACEI treatment reduced the immunoexpression of ICAM-1 in RAECs cultured in a normoglycemic medium with ox-LDL but not in a hyperglycemic medium. In addition, the MCP-1 expression was reduced in cells that were incubated with medium containing low glucose levels independent of ACEI treatment.

Several experimental studies with hypercholesterolemic rabbits have demonstrated that the use of ACEI reduces the development of atherosclerotic lesions36,37. The proposed mechanism for the benefit of ACE inhibitors is related to the activity of cytokines to mediate inflammatory responses38 and increase the expression of vascular adhesion molecules. Hernandez-Presa et al.39 have demonstrated that the use of quinapril in hypercholesterolemic rabbits prevents the activation of NF-kB, which is a key factor in controlling the expression of MCP-1.

ACE may be associated with the development and vulnerability of atherosclerotic plaques by directly regulating inflammatory cells. Angiotensin II promotes the recruitment of monocytes and lymphocytes, increasing the expression of TNF-α, IL-6 and Cox-2 in atherosclerotic arteries39.

We have previously conducted a study using an in vivo model with hypercholesterolemic and hyperglycemic rabbits that were treated with ACEI. Our model has shown that the immunohistochemical expression of ICAM-1 and MCP-1 in the intima of aorta is significantly lower in the hypercholesterolemic and normoglycemic groups that were treated with ACEI compared to hypercholesterolemic and hyperglycemic groups (in press). These results suggest that ACEI treatment influences the expression of ICAM-1 and MCP-1 to mediate the inflammation in the early stages of atherogenesis, and the efficacy of ACEI is closely related to the glycemic effects on macrovascular disease.

The presence of ACEI reduced the immunoexpression of ICAM-1 in RAECs cultured in medium with ox-LDL and 5.5 mM of glucose. This reduction was not observed when cells were cultured in medium containing high glucose levels (22.2 mM). These results reinforce the need for glycemic control to optimize the benefits of ACEI treatment.

In addition to its vasoconstrictor activity, angiotensin II increases the rolling, adhesion and migration of leukocytes by directly regulating vascular pro-inflammatory mediators and acts as a strong modulator of the production of reactive oxygen species in blood vessels. Angiotensin II stimulates NADPH oxidase, the expression of ICAM-1 and macrophage infiltration independent of blood pressure elevation40. ACEI and the antagonist of the AT1 receptor of angiotensin II inhibit the renin-angiotensin system and increase bradykinin levels41. Some of the beneficial effects are attributed to changes in the levels of...
angiotensin II and bradykinin and the activation of distinct signaling cascades. Reduced immunohistochemical expression of MCP-1 was observed in cells that were incubated with low glucose independent of ACEI treatment, suggesting that the concentration of glucose may be a critical factor for the expression of MCP-1. This result supports the findings of Takaishi et al., suggesting that hyperglycemia accelerates the production of MCP-1 through a signaling pathway that is sensitive to reactive oxygen species and p38 MAPK in endothelial cells. We observed protective effects, which were indicated by decreased ICAM-1 in the presence of ACEI and reduced MCP-1 when low glucose levels were present. Therefore, we hypothesized that separate mechanisms may differentially regulate the expression profiles of ICAM-1 and MCP-1, and the combination of these mechanisms with reduced blood glucose levels and ACE inhibition may contribute to the protection of endothelial cells.

Human atherosclerosis is associated with DNA damage in circulating cells and those within the vessel wall. Although atherosclerotic plaques develop as a chronic inflammatory reaction, DNA damage in cells within the lesion may play an important role in atherogenesis and the behavior of established lesions. DNA damage frequently occurs in cells that are exposed to oxidative stress, and increased oxidative stress may initiate lipid peroxidation in cell membranes, induce the damage of membrane proteins or cause DNA fragmentation. In diabetes, hyperglycemia induces superoxide generation in endothelial cells causing oxidative stress with atherogenic effects. Ox-LDL and hyperglycemia may induce the production of reactive oxygen species in the mitochondria of macrophages and endothelial cells. Hyperglycemia enhances free radical production, inducing oxidative damage that is an important enhancer of the progression of atherosclerosis.

The current study did not show increased DNA damage in endothelial cells that were cultured in medium with high concentrations of glucose. In addition, ACEI treatment did not confer protection against DNA damage. The period of incubation with high glucose concentrations may not have been sufficient to induce DNA damage, which was detected using the comet assay, and to influence ACEI activity. Previous studies have shown that intermittent high glucose levels cause more damage than a constant high glucose concentration in cultured umbilical vein endothelial cells.

A number of studies have shown that angiotensin II induces oxidative stress in endothelial cells and that ACEI reduces the production of reactive oxygen species in pathological conditions. In a study with endothelial cells cultured in vitro, the ACE inhibitors, temocapril and captopril, attenuated oxidative stress-induced endothelial cells apoptosis. An angiotensin II receptor blocker, telmisartan, modulates inflammatory and oxidative damage in cultured human umbilical vein endothelial cells.

In conclusion, the current study highlighted the protective effects of RAS inhibition in the control of developing atherosclerosis. We did not observe any changes in the amounts of DNA damage. However, the ACEI showed protective effects by reducing inflammatory cytokine expression, and this protection was dependent on adequate glycemic regulation.

Conflict of interest
No conflict of interest.

Acknowledgement
Daniel Pomaro was supported by CNPq, a Brazilian foundation.

References
5. Martinet W, Knaapen MW, De Meyer GR, Herman AG, Koekx MM. Elevated levels of oxidative DNA damage and DNA repair enzymes in human atherosclerotic plaques. Circulation. 2002;106(8):927-932. doi: 10.1161/01.cir.0000026393.47805.21


44. Pockley P. Clean-up strategy at Australian nuclear site called into question. *Nature.* **2000**;404(6780):797. doi: 10.1038/35009207


