in vivo *24*: 147-152 (2010)

Chronic Hind Limb Ischemia Reduces Myocardial Ischemia-Reperfusion Injury in the Rabbit Heart by Promoting Coronary Angiogenesis/Arteriogenesis

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Abstract. *Background/Aim: Application of ischemic injury in a remote organ may provide protection of other tissues against ischemia. We hypothesized that ischemia in the rabbit hind limb protects against myocardial ischemia by increasing angiogenesis/arteriogenesis. Materials and Methods: In the first experiment, severe limb ischemia (LI) was induced in 26 New Zealand White rabbits by excision of the femoral artery while another 26 served as controls (no ischemia; sham operation [SHO]). Four weeks later, the blood vessels of the subendocardial and intramyocardial areas of the excised hearts were counted. In the second experiment, 14 LI rabbits and 14 SHO controls were subjected to 30 min of regional heart ischemia and 3 h reperfusion. Infarct size and the areas-at-risk were determined. Results: Compared with controls, LI rabbits showed more subendocardial (103±14 vs. 113±13 capillaries/mm2, respectively; p=0.01) and intramyocardial blood vessels (102±12 vs. 114±16 capillaries/mm^2, respectively; p=0.009). LI rabbits had significantly smaller infarct size compared with the SHO animals (infarct areas/areas-at-risk: 14.37±11.23% vs. 31.31±13.73%, respectively; p=0.003). Conclusion: Chronic hind LI reduces myocardial infarct size by promoting coronary angiogenesis/arteriogenesis in an experimental model.*

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Key Words: Chronic hind limb ischemia, angiogenesis, ischemia reperfusion injury, remote ischemic preconditioning, infarct size, arteriogenesis.

Several strategies have been developed for the protection of organs from ischemia-reperfusion injury (IRI), referred to as ischemic preconditioning (IPC) (1-3). IPC describes the method by which the target organ is conditioned prior to the ischemic insult to reduce the extent of the IRI (1-3). Briefly, the mechanism involves a brief direct ischemic insult to the target organ followed by blood reperfusion; as a result, the target organ is made tolerant to IRI (1-3).

In the mid and late 1990s, a novel method of IPC was described. A brief period of ischemia in a remote organ was found to reduce myocardial infarct size protecting against subsequent sustained myocardial ischemia. This model was termed remote ischemic preconditioning (4, 5). Limb IPC was shown in animal studies to reduce myocardial infarct size (6-11).

Chronic limb ischemia (LI) results in the development of collateral circulation to maintain adequate blood supply (12, 13). Two distinct forms of adaptive response by the vascular system have been identified. Arteriogenesis refers to the growth and enlargement of preexisting collaterals (14). The main stimulus that stimulates arteriogenesis is the increased shear stress inside preexisting collateral vessels and small arteries proximal to a point of severe or total obstruction (15). Angiogenesis refers to the increase in capillary density in ischemic tissues (16). Angiogenesis seems to be primarily regulated by humoral stimuli, related to the hypoxic environment that develops in the setting of tissue ischemia (15, 17). In the rabbit model of hind LI, endogenous vascular remodeling is accomplished through angiogenesis and/or arteriogenesis (18-21).

We hypothesized that chronic LI (possibly a more exaggerated model of remote transient ischaemia) can act as a signal to promote angiogenesis/arteriogenesis in distally located tissues (*e.g.* the heart).

Materials and Methods

The experiments were carried out on 80 New Zealand White rabbits weighing 2.8-3.5 kg each. All animals were housed in individual cages, in a climate-controlled environment and an artificial 12-h light-dark cycle. All animals received humane care in compliance with the "Guide for the Care and Use of Laboratory Animals" (22). Access to food and water was unrestricted. The experimental protocol was approved by the Athens Committee of Animal Care.

Preparation and induction of femoral ischemia in the rabbit model. The rabbits were randomly divided into two groups (52 and 28 animals). In each group, severe permanent ischemia of the right hind limb was surgically induced in half of the animals according to a previously described model (16), while the other half were sham operated (SHO).

In brief, animals were anesthetized with a mixture of ketamine (50 mg/kg) and acepromazine (0.8 mg/kg) after premedication with xylazine (2.5 mg/kg). A longitudinal incision was performed extending inferiorly from the inguinal ligament to a point just proximal to the patella. The femoral artery was dissected free along its entire length; all branches of the femoral artery (including the inferior epigastric, deep femoral, lateral circumflex, and superficial epigastric arteries) were also dissected free. The popliteal and saphenous arteries distally were further dissected. All the above arteries as well as the external iliac artery proximally were ligated (4.0 silk; Ethicon, Sommerville, NJ, USA). Finally, the femoral artery was completely excised from its proximal origin to its bifurcation distally (to the saphenous and popliteal arteries). Postoperatively, all animals were closely monitored. Analgesia was administered subcutaneously as required by evidence of discomfort throughout the study duration. Prophylactic antibiotics were also administered subcutaneously for a total of 5 days postoperatively. Animals in the control group were sham operated, receiving identical anesthesia and antibiotic regimen, but with no ischemia induced. All animals were left to recover under close supervision and proper housing and care.

At the end of the fourth week, all animals were macroscopically examined for distal LI (skin necrosis or limb atrophy). At the same time, the calf blood pressure was measured in both hind limbs using a Doppler flow meter with an 8 MHz transducer (Direction volume meter, DVM 1200, Hadeco, USA). On each occasion, the rabbits were sedated and both hind limbs were shaved and cleaned. The pulse of the posterior tibial artery was detected using a Doppler probe, and the systolic blood pressure in both limbs was determined using standard techniques. The calf blood pressure ratio was defined for each rabbit as the ratio of the systolic blood pressure of the ischemic limb to that of the normal limb (14, 16).

Heart excision and histological preparation. Four weeks after the induction of ischemia or the sham operation, all animals were euthanized (by injecting an overdose of anesthetics) and the hearts were excised. After flushing with cold normal saline, the heart was fixed in 10% buffered formalin solution. Full-thickness tissue sections of the myocardium were removed the following day, including the free wall of the left ventricle and the intraventricular septum. The sections were obtained from the same area of the heart of each animal (the upper part of the papillary muscle) and were subsequently embedded in paraffin.

Tissue sections of 3 μm thickness were cut onto silane-coated slides and stained with hematoxylin and eosin for routine histopathological examination. Separate slides were stained for the immunohistochemistry investigation with peroxidase using smooth muscle actin (clone 1A4, Lab Vision, USA) as a primary antibody for the detection of small-sized vessels (non-capillary, non-lymphatic). Since capillaries and lymphatics lack smooth muscle wall, by use of this method it is possible to identify pre-capillary arterioles and post-capillary venules (18).

After dewaxing, tissue sections were placed in a glass rack and put into a 0.1 M sodium citrate buffer. For antigen retrieval, the slides were heated by microwave (Proline Powerwave 800) for 2-4 min at 700 W and left to cool for 20 min. The slides were washed 3 times in Tris-buffered saline before application of the primary antibody. Immunohistochemistry was subsequently performed using a LabVision autostainer 480, with ultravision/HRP mouse DAB kit (TM-015-HD).

Blood vessel count. Ten different fields from the subendocardial area and an additional 10 from the intramyocardial area were randomly selected. The number of non-capillary non-lymphatic blood vessels was counted using a ×20 objective (mean number of non-capillary, non-lymphatic blood vessels/mm^2). Large-sized arteries or venules (the immediate branches of the epicardial coronary network) were not included. The two observers were blinded to treatment in order to avoid potential observer bias. The agreement between the two observers was 95%.

Myocardial ischemia-reperfusion procedure. The rabbits of the second group were subjected to sustained ischemia of the heart for 30 min followed by 3 h reperfusion according to a previously described model (23). Briefly, the rabbits were anesthetized by injecting 30 mg/kg sodium thiopeptone (Pentothal, Abbott) slowly into an ear vein. Mechanical ventilation was performed by intubation through a midline tracheal incision. A positive pressure respirator for small animals (MD Industries, Mobile, AL, USA) was used and was adjusted at a rate to keep arterial blood gases within the standard values. The carotid artery was catheterized for continuous blood pressure monitoring *via* a transducer connected to a multichannel recorder. The chest was opened *via* a left thoracotomy in the fourth intercostal space. After pericardiotomy, the beating heart was exposed. A 3-0 silk thread was passed through the myocardium around a prominent branch of the left coronary artery. Ischemia was induced by pulling the ends of the suture through a small segment of a soft tube, which was firmly attached against the artery with a clamp. Myocardial ischemia was maintained for 30 min. The successful induction of ischemia was verified by a blood pressure reduction on the recorder and by visual inspection (cyanosis) of the heart. Myocardial reperfusion lasting for 180 min was achieved by releasing the clamp.

Risk area and infarct size measurement. After the end of the reperfusion period, all hearts were harvested, mounted on a reperfusion apparatus and perfused (50 mmHg) retrogradely *via* the aorta with normal saline (10 ml/min) at room temperature for 2 min. When all residual blood had been removed from the coronary arteries, the coronary ligature was retightened at the same site and 5 ml of green fluorescent microspheres (2-9 μm diameter; Duke Scientific Corp., Palo Alto, CA, USA) suspended in saline were infused over 5 min for the delineation of the normally perfused tissue from the risk zone. The hearts were frozen at –20˚C for 24 h and were then sliced into 3 mm sections from the apex to the base. The slices were

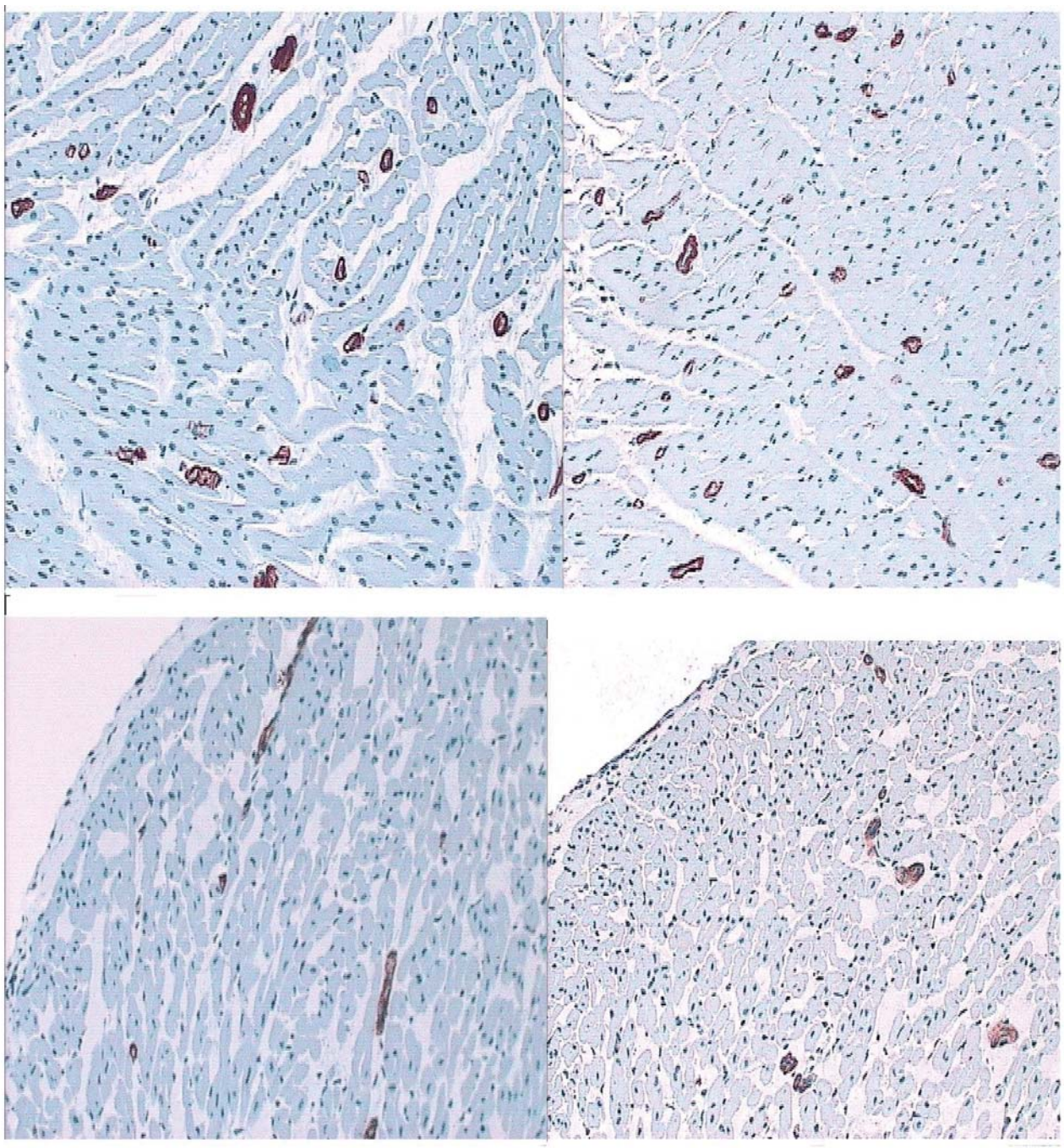

Figure 1. *Upper panel: Sections from a non-ischemic normal heart of a rabbit undergoing chronic hind limb ischemia, showing increased number of non-capillary, non-lymphatic blood vessels. Lower panel: Sections from a non-ischemic normal heart of a rabbit that underwent sham operation for hind limb ischemia, showing low density of non-capillary, non-lymphatic blood vessels.*

incubated in 1% triphenyl tetrazolium chloride (TTC) in isotonic phosphate buffer solution, pH 7.4, for 20 min at 37˚C. TTC reacts with dehydrogenase enzymes and nicotinamide adenine dinucleotide in viable tissue; the infarcted area was defined as the area which did not stain. The heart slices were immersed in 10% formaldehyde solution for 24 h to delineate the infarcted areas more clearly. The slices were then pressed between glass plates and were examined under UV light (λ=366 nm) to identify the borders between the risk zone and the normal area. The infarcted areas, the areas-at-risk and the normal areas were traced onto an acetate sheet that was placed over the top glass plate. The tracings were subsequently scanned into Adobe Photoshop 6.0 and measured with the Scion Image program

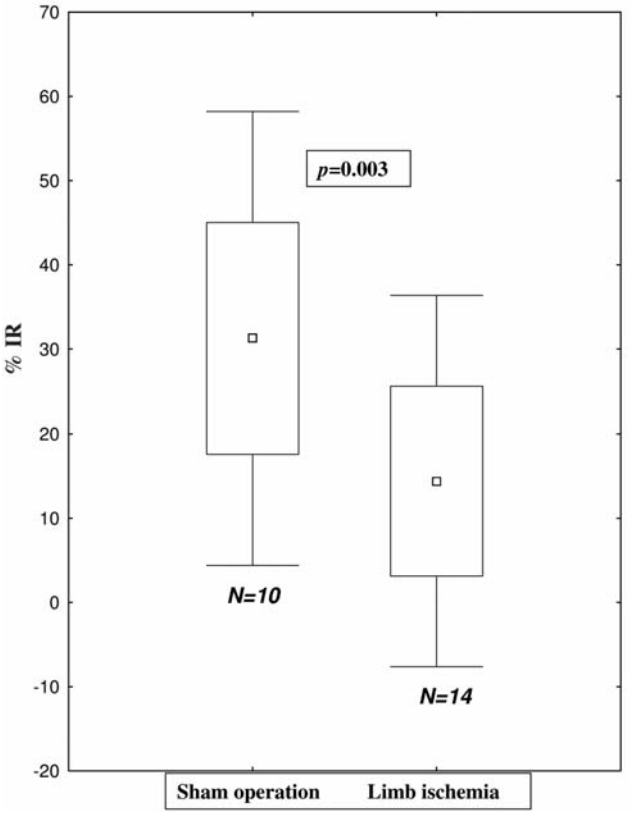

Figure 2. *A plot of the percentage ratio of infarct areas/areas-at-risk size for sham operated and limb ischemic rabbits.*

(Scion Inc., Frederick, Maryland, USA). The areas-at-risk were automatically transformed into volumes by multiplying the corresponding areas by thickness (3 mm). The volume of infarct areas and areas-at-risk were expressed in cubic centimeters (cm^3) and the percentage ratio of infarct areas/areas-at-risk (%I/R) was calculated.

Statistical analysis. Values are given as the mean±standard deviation. Differences in numbers of vessels and %I/R were compared using Student's *t*-test. A *p*-value <0.05 was considered significant.

Results

Severe LI and atrophy were induced in all LI rabbits. The ratio of ischemic/normal limb blood pressure was lower in all the LI rabbits; none of the SHO animals demonstrated any limb ischemia, atrophy or decrease in limb blood pressure (ratio of ischemic/normal limb blood pressure: 0.51±0.16 *vs*. 1.12±0.22 for the LI and SHO animals, respectively; $p<0.001$). Seven LI rabbits of the first group died after femoral artery excision; 4 SHO animals of the second group died due to hemodynamic instability or other technical reasons. Thus, a total of 69 rabbits completed the study: 19 LI and 26 SHO animals from the first group, 14 LI and 10 SHO animals from the second group.

No differences were observed in the heart weight between animals in the LI compared with the SHO groups (7.03±0.3 *vs*. 7.26±0.3 g; $p=0.446$). Similarly, no differences were observed in the heart rate at baseline (267±9 *vs*. 270±6 beats/min, respectively; $p=0.782$), during the 30 min of ischemia (260±6 *vs*. 263±12 beats/min, respectively; $p=0.881$), or during the 3h of reperfusion (243±14 *vs*. 237±12 beats/min, respectively; $p=0.639$). Finally, no differences were observed in the average blood pressure values during the same time intervals (74±4 *vs*. 78±5 mmHg, respectively; $p=0.521$; 70±3 *vs*. 67±5 mmHg, respectively; $p=0.711$; and 66±4 *vs*. 67±4 mmHg, respectively; $p=0.865$).

Blood vessel count. There were more non-capillary, non-lymphatic blood vessels in the subendocardial (113±13 *vs*. 103±14 capillaries/mm^2, respectively; $p=0.01$) and intramyocardial area (114±16 *vs*. 102±12 capillaries/mm^2, respectively; $p=0.009$) in the LI compared with the SHO group (Figure 1).

Infarct size and area-at-risk measurement. LI rabbits had significantly smaller infarct size compared with the SHO animals (%I/R: 14.37±11.23% *vs*. 31.31±13.73%, respectively; $p=0.003$; Figure 2).

Discussion

The present study suggests that induction of severe ischemia in the rabbit hind limb may be associated with a reduced myocardial infarct size *via* a mechanism involving coronary angiogenesis/arteriogenesis.

Our data support the conclusions reached in previous studies (6-11) showing that the induction of severe LI is associated with a reduction in myocardial infarct size compared with animals without previous LI. Several mechanisms have been proposed to account for this effect including inflammatory gene suppression (6), modulation of K^+-ATP channels (7, 9), nuclear factor kappa-B p105 and inducible nitric oxide synthase (8), adrenergic (10) or free radical (11) pathways.

Our findings suggest that the protective effect on the rabbit heart induced by severe LI may be associated with the promotion of coronary angiogenesis/arteriogenesis *via* remote ischemic preconditioning. Chronic LI promotes the development of collateral blood vessels to compensate for ischemia and provide adequate blood supply (12, 13). The development of collateral circulation during chronic LI involves the release of several growth factors (such as the vascular endothelial growth factor [VEGF] and the basic fibroblast growth factor [bFGF]) (24-26). As these growth factors are released in the systemic circulation, they may exert their effects on other arterial beds, such as the coronary circulation. This mechanism may explain the increased blood

vessel count in the LI compared with the SHO animals. *Via* the model of remote ischemic preconditioning, chronic LI results in angiogenesis/arteriogenesis in the normal heart. In turn, the increased number of blood vessels may account for the reduced myocardial infarct size. The mechanism underlying this process may be the ischemia-induced increased collateral circulation.

An earlier report investigated the processes of angiogenesis and arteriogenesis in the rabbit hind limb after induction of ischemia (removal of femoral artery) (27). Five days following the induction of ischemia, there was a significant increase in the adductor muscle capillary density (from 216±24 to 351±24 capillaries/mm^2; $p<0.05$), reflecting an increased rate of angiogenesis following the induction of ischemia. Similarly, 10 days following the induction of ischemia, there was a considerable increase in the density of collateral vessels, reflecting an increased arteriogenesis.

Nonischemic chest pain occurs in almost half of all patients undergoing percutaneous coronary intervention (PCI) (28-30). It was hypothesized that the reason for this chest pain is local vessel injury (stretch pain or vasospasm) induced by dilation of the coronary lesion/coronary stent implantation (28-30). This chest pain is often accompanied by electrocardiographic changes and elevation of cardiac enzyme levels (troponin I and creatine kinase-MB) (28-30). Upon subsequent dilatations, the ECG changes and anginal severity subside. If acute remote IPC exists and can be reproduced, perhaps compression of a major limb artery during primary PCI for S-T elevation myocardial infarction may reduce myocardial reperfusion injury.

A recent randomized study investigated the effect of IPC on cardiac troponin I release after elective PCI (31). Patients scheduled for elective PCI with undetectable pre-procedural cardiac troponin I levels were randomized to receive either remote IPC induced by three 5 min inflations of a blood pressure cuff around the upper arm to 200 mmHg followed by 5 min intervals of reperfusion or no remote IPC (an uninflated arm cuff) before the procedure. The median troponin I levels 24 h after PCI was lower in the remote IPC compared with the control group (0.06 *vs*. 0.16 ng/ml; $p=0.040$) (31). More patients receiving remote IPC had troponin I levels <0.04 ng/ml compared with the control group in (44 *vs*. 24 patients, or 42% *vs*. 24%; $p=0.01$). Furthermore, the individuals who received remote IPC experienced less chest discomfort ($p=0.0006$) and ECG ST-segment deviation ($p=0.005$) compared with controls. Finally, the major adverse cardiac and cerebral event rate at 6 months was lower in the remote IPC compared with the control group (4 *vs*. 13 events; $p=0.018$) (31). Thus, it appears that remote IPC is associated with multiple beneficial effects in these patients. Future studies will define the effects of remote IPC in patients experiencing a myocardial infarction or undergoing PCI.

A limitation of our study is that hind LI cannot fully mimic remote preconditioning; rather, hind LI is a stress stimulating the body's compensatory mechanisms which include angiogenesis/arteriogenesis in the hind limb, as well as remote coronary angiogenesis/arteriogenesis. Additionally, we did not measure cardiac enzyme changes during our experiment to assess cardiac damage. Finally, we did not measure local and systemic levels of various vascular growth factors (*e.g*. VEGF or bFGF), mRNA or protein levels.

In conclusion, the present study shows that chronic hind LI leads to coronary angiogenesis/arteriogenesis which may be associated with reduced infarct size following myocardial ischemia and reperfusion. Future studies may extend our model of hind LI to identify the relevant mediators responsible for increased angiogenesis and arteriogenesis occurring in remote organs.

Acknowledgements

This study was supported by a grant from the Hellenic Cardiological Society and the Cardiovascular Research Institute, Ioannina and Athens, Greece (VV and KK).

Conflict of Interest

None declared.

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Received September 6, 2009
Revised February 18, 2010
Accepted February 22, 2010