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*ORIGINAL ARTICLE*

# Effect of Chronic High Altitude Hypoxia on Foetal and Maternal Juxta-Alveolar Distal Pulmonary Smooth Muscle Cells Actin and Calponin Organisation and Growth Profiles

## *Effets De L'hypoxie Chronique Liee A La Haute Altitude Sur Les Cellules Musculaires Lisses Pulmonaires Distales Juxta-Alveolaires, Organisation Des Filaments D'actine Et De Calponine Et Leur Profil De Croissance*

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**ABSTRACT**

BACKGROUND: **The effect of chronic high altitude hypoxia (CHAH) in the juxta-alveolar region near the air-blood interface is unknown because of the experimental inaccessibility of this region.**

OBJECTIVE: **To examine primary cultures of digested juxta-alveolar smooth muscle cells for hypoxia-induced changes.**

METHODS: **Smooth Muscle Cells (SMCs) obtained by dispase digestion of the extreme lung parenchyma were used to study the effect of CHAH in the juxta-alveolar region and foetal and maternal cells were compared. Pulmonary venous SMCs were also obtained from dissected 5th to 7th generation levels pulmonary veins (<0.5 mm). Fluorescence tagged antibodies against alpha smooth muscle actin (alpha SMA) and calponin respectively were used as markers to identify cellular structural differences by routine immunohistochemistry. Comparison of the functional integrity of the cells was made using their growth profiles obtained by radiolabeled thymidine incorporation and liquid scintillation counting.**

RESULTS: **Marked differences were seen in juxta-alveolar SMCs obtained by digestion of extreme lung parenchyma of hypoxic sheep. Hypoxic adult sheep cells showed increased filamentation. Hypoxic foetal sheep cells showed internal restructuring and disorganization of both alpha-SMA and calponin filaments. The growth profiles of juxta-alveolar SMCs showed that the hypoxia-affected cells of both the foetus and adult sheep had a fast initial growth rate peaking at 48h while their normoxic equivalents had a steadier growth rate peaking at 72h. Hypoxia-affected cells showed contact inhibition at ~50% subconfluence and apoptosis by 48h.**

CONCLUSION: **Chronic high altitude hypoxia causes both phenotypical and functional changes in pulmonary smooth muscle cells near the air/blood interface. WAJM 2010; 29(6): 388–392.**

Keywords: **Chronic high altitude hypoxia, pulmonary smooth muscle cells, lung, foetus.**

***RÉSUMÉ***

***CONTEXTE:*** *l'effet de l'hypoxie chronique liée à la haute altitude (HCHA) dans la région juxta-alvéolaire proche de l'interface air-sang est inconnu du fait de l'inaccessibilité de cette zone aux études expérimentales.*

***METHODES:*** *Des cellules musculaires lisses (CML) obtenues à partir d'un broyat de parenchyme pulmonaire périphérique ont été utilisées pour étudier l'effet de l'HCHA. Les prélèvements ont concerné aussi bien des ovins adultes femelles porteuses que des fœtus pour permettre de comparer les effets biologiques. Les CML des veines pulmonaires ont été obtenues par dissection de la 5ème à la 7ème subdivision des veines pulmonaires (diamètre<0,5 mm). Des anticorps anti-actine alpha et anti-calponine marqués à la fluoroscéine ont été utilisés pour identifier les différences structurelles par méthode immunohistochimique classique. La comparaison de l'intégrité fonctionnelle et des profils de croissance des CML a été faite par scintigraphie à la thymidine marquée.*

***RESULTATS:*** *nous avons retrouvé d'importantes modifications morphologiques sur les CML obtenus par broyat de parenchyme pulmonaire périphérique d'ovins en hypoxie chronique. En effet, chez les sujets adultes, les CML ont montré une augmentation de la filamentation. Chez les fœtus, elles ont subi une restructuration et une désorganisation des filaments d'actine alpha et de calponine. Les profils de croissance ont également subi des modifications fonctionnelles liées à l'hypoxie chronique aussi bien chez les sujets adultes que les fœtus. La croissance était plus rapide chez les CML hypoxiques avec un pic atteint en 48h contrairement aux CML normoxiques où le pic était atteint en 72h. Par ailleurs, les CML hypoxiques ont montré une baisse du contact cellulaire dans presque 50% des cas avec une apoptose au bout de la 48ème heure.*

***CONCLUSION:*** *l'hypoxie chronique liée à la haute altitude entraine des modifications aussi bien phénotypiques que fonctionnelles des cellules musculaires lisses pulmonaires proches de l'interface air-sang.* ***WAJM 2010; 29(6): 388–392.***

***Mots clé:*** *Hypoxie chronique liée à la haute altitude, cellules musculaires lisses, poumons, fœtus.*

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**Abbreviations: SMC,** Smooth muscle cell; **CHAH,** Chronic high altitude hypoxia; **alpha SMA,** alpha Smooth muscle actin; **eNOS, endothelial** Nitric oxide synthase; **mRNA,** messenger Ribonucleic acid; **RT-PCR,** Reverse transcription polymerase chain reaction; **NO,** Nitric oxide; **vWF,** von Willebrand factor.

## INTRODUCTION

Murata *et al*[1] showed that hypoxia (5% oxygen, for seven days) impaired endothelium-dependent relaxation of intrapulmonary arteries but not of mesenteric arteries from rabbits. These workers showed that hypoxia did not change the endothelial nitric oxide synthase (eNOS) messenger riboneucleic acid (mRNA) expression, eNOS protein expression, or caveolin expression in the endothelium assessed by semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) or whole mount immunostaining. They suggested that the effect of hypoxia on the endothelium was not due to less nitric oxide (NO) being produced or more NO being scavenged by adding L-arginine, tetrahydropterin or superoxide dismutase. They further observed that the hypoxic endothelium showed atrophy of endothelial cells. They suggested that chronic hypoxia caused changes in endothelial cell structure and organisation rather than changes in eNOS expression in the endothelium or NO sensitivity of smooth muscle cells.

The effect of oxygen tension on the structure and function of cells of the air/blood interface is difficult to examine because of the difficulty of experimental accessibility of this portion of the pulmonary architecture in its intact form. We generally can dissect down to about the seventh generation vessels or bronchioles whereas the vascular and bronchiolar trees branch down to about 28 generations till they reach the alveoli. Microvascular smooth muscle cells responsible for vascular reactivity as well as microbronchiolar smooth muscle cells responsible for bronchiolar reactivity near the air/blood interface of the lung may undergo structural changes under chronic hypoxia. Chronic hypoxia is thought to contribute to the etiology of neonatal and adult pulmonary hypertension and other pulmonary conditions.[2] In the present study we compared foetal and maternal pulmonary smooth muscle cells similarly isolated from distal lung parenchyma of chronically hypoxic pregnant sheep (foetus and mother). We examined the structural expression of alpha smooth muscle actin and calponin by qualitative immunohistochemistry and compared the tritiated iodine incorporation growth profiles of the foetal and maternal cells to determine some phenotypical and functional effects of chronic high altitude hypoxia on smooth muscle cells close to the air/blood interface.

## MATERIALS, AND METHODS

Freshly excised lungs were obtained from pregnant sheep kept at The White Mountain Research Station, Bishop, California, USA, at 3,801m altitude (~12, 470 ft) and $PaO_2$ 60 ± Torr from 35 days to 145 days of gestation, term being 147 days and from their age matched controls kept at sea level. Extreme lung parenchyma were obtained by excising 0.5 mm thick strips from the lung edges. These were digested in 2 U dispase/ml phosphate buffered saline at 35 C over 30 min under gentle agitation. Sterile conditions were used and cells were cultured as earlier described by John *et al*.[3] The smooth muscle cells obtained by dispase digestion of the extreme parenchyma were used to study the effect of chronic hypoxia in the juxta-alveolar region, i.e. closest to the air/blood interface and the foetal and maternal cells were compared. Pulmonary venous smooth muscle cells were also obtained from dissected $5^{th}$ to $7^{th}$ generation levels intrapulmonary veins (<0.5 mm ) of the sheep lungs. Cultures were prepared so that groups for comparison were in the same experimental conditions to limit in-between group extraneous factors. Monoclonal antibodies against alpha smooth muscle actin (alpha-SMA) and calponin respectively were used as markers to identify some structural differences in the cells. Routine immunohistochemistry with slide chambers was used to label these proteins as earlier published by John *et al*.[4] A comparison of the functional integrity of the cells was made by comparing their growth profiles obtained by radiolabeled thymidine incorporation using six well clusters per group.[5,6] Subconfluence (~10% confluence) seeding of cells at a density of 10,000 cells/100 ul/well in growth medium was done.[6] On the next day, the wells were washed thrice with phosphate buffered saline and then incubated with 1% foetal bovine serum for 24 h for $G_0$ synchronization. The medium was replaced with feed medium containing tritiated thymidine (1 uCi/well).[6] Cells were incubated and then harvested at various times, 24h, 48h, 72h, 96h post thymidine administration. Radiolabeled thymidine incorporation was determined by liquid scintillation counting.[6]

## RESULTS

Cells cultured were shown to be smooth muscle cells by the presence of alpha smooth muscle actin and the lack of von Willebrand factor (vWF, endothelial cell marker). Since the juxta-alveolar smooth muscle cells obtained by digestion could not be characterized as venous, arteriolar or bronchiolar, the smooth muscle cells from dissected intrapulmonary veins were used as standards for comparison. Figures 1 and 2 show that both the foetal and maternal venous smooth muscle cells from normoxic animals had alpha smooth muscle actin and calponin respectively. Smooth muscle cells obtained from the same animals by dispase digestion of the extreme lung parenchyma (juxta-alveolar smooth muscle cells) had similar expressions of these proteins (not shown) indicating that the normal cells were similar up and down the bronchiolar tree. For the hypoxic animals, dissected vein cells were similar to those of normoxic animals. For the hypoxic animals, marked differences were seen in juxta-alveolar smooth muscle cells (Figure 3 and 4) obtained by digestion of extreme lung parenchyma. For hypoxic adult sheep these cells showed increased filamentation (Figure 3 and 4). For the hypoxic foetal sheep these cells showed internal restructuring and dis-organisation of both actin and calponin filaments (Figures 3 and 4). The growth profiles of juxta–alveolar smooth muscle cells showed that the hypoxia-affected cells of both the foetus and adult sheep had a fast initial growth rate peaking at 48h while their normoxic equivalents had a steadier growth rate peaking at 72h (Figure 5). Hypoxia-affected cells showed contact inhibition at ~50% subconfluence and apoptosis by 48h (Figure 5).

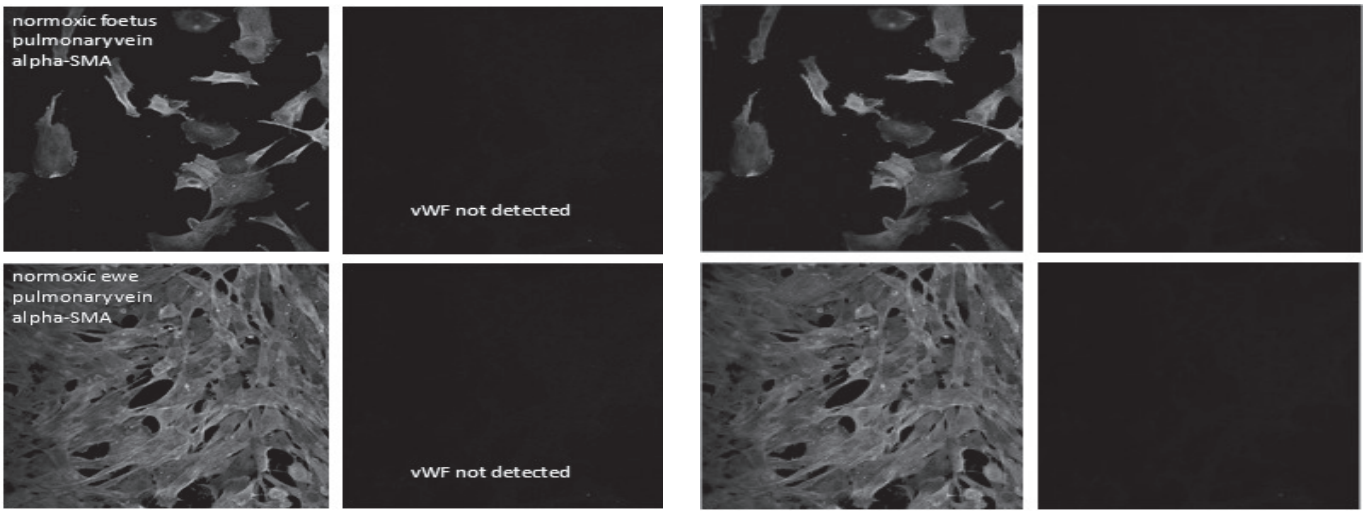


**Figure 1: Fluorescence micrographs of ovine intrapulmonary vein smooth muscle cells tagged with alexa-488-labeled monoclonal antibody against alpha smooth muscle actin. Upper panels show foetal cells, lower panels show cells of the maternal ewe. Second and fourth columns show failed tagging with alexa-488 labeled anti-von Willebrand factor (endothelial cell marker) in identical monolayers.**

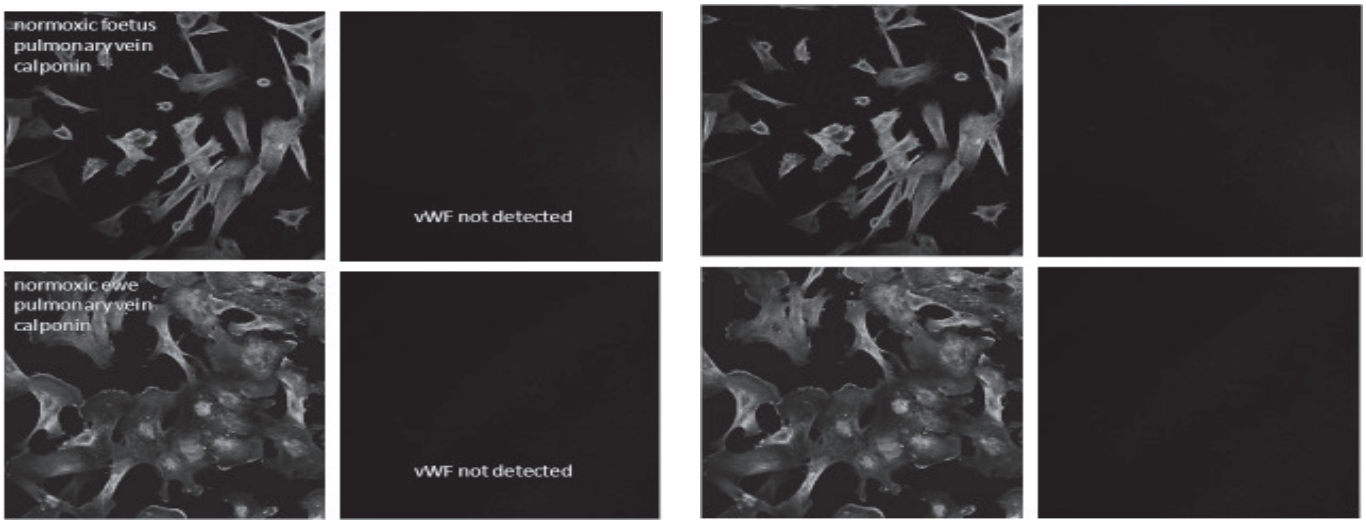


**Figure 2: Fluorescence micrographs of ovine intrapulmonary vein smooth muscle cells tagged with alexa-488-labeled monoclonal antibody against calponin. Upper panels show foetal cells, lower panels show cells of the maternal ewe. Second and fourth columns show failed tagging with alexa-488 labeled anti-von Willebrand factor (endothelial cell marker) in identical monolayers.**

## DISCUSSION

The present study shows that chronic high altitude hypoxia causes both phenotypical and functional changes in pulmonary smooth muscle cells near the air/blood interface. While the phenotypical effects of chronic hypoxia is different in foetal cells (protein disorganisation) and adult cells (increased filamentation) (Figures 3 and 4), the hypoxia effect on the cell growth patterns was similar for both the foetus and adult cells (Figure 7). Extreme lung parenchyma cells from the foetal lungs appear to be internally disorganized after long exposure to chronic hypoxia (Figures 3 and 4). These cells have not experienced normoxia as the foetal lung is non-breathing and the cells appear to lack adaptive mechanisms to abnormal oxygen tension compared with equivalent adult cells. Chronic hypoxia has been associated with neonatal diseases such as persistent pulmonary hypertension of the newborn and respiratory distress syndrome.[7,8] The present study reveals that smooth muscle cells closer to the air/blood interface are especially vulnerable to chronic hypoxia. In previous work we showed that oxygen tension alters intrapulmonary vascular endothelial function by altering the relationship between caveolin-1 and nitric oxide synthase.[3] Other studies have shown effects of hypoxia on nitric oxide mediated reactivity of intrapulmonary veins and arteries[9,10,11] or on remodeling of intrapulmonary vessels.[12,13] Following the observation of Murata *et al.,* (2001)[1] which suggests a stronger effect of hypoxia on vascular remodeling than on nitric oxide mediated vascular reactivity of intrapulmonary vessels, the present study reveals that pathological changes beyond the intrapulmonary vessels, at the extreme end of the pulmonary or bronchiolar trees and alveolar regions, may also be a major cause of disease induced by chronic hypoxia.

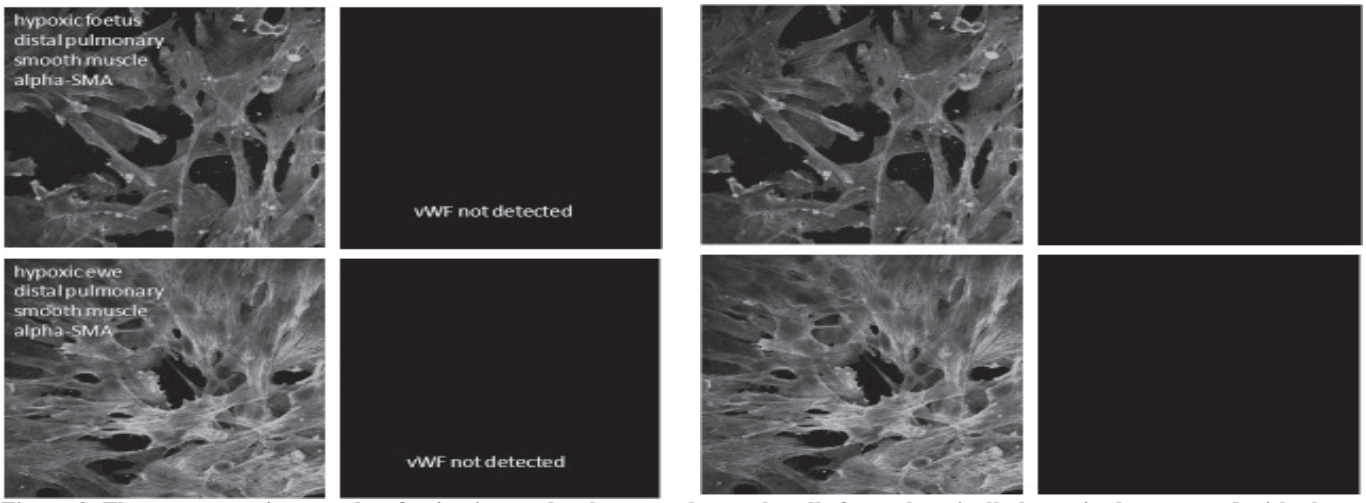


**Figure 3: Fluorescence micrographs of ovine juxta-alveolar smooth muscle cells from chronically hypoxic sheep tagged with alexa-488-labeled monoclonal antibody against calponin. Upper panel shows foetal cells, lower panel shows cells of the maternal ewe. Second and fourth columns show failed tagging with alexa-488 labeled anti-von Willebrand factor (endothelial cell marker) in identical monolayers.**

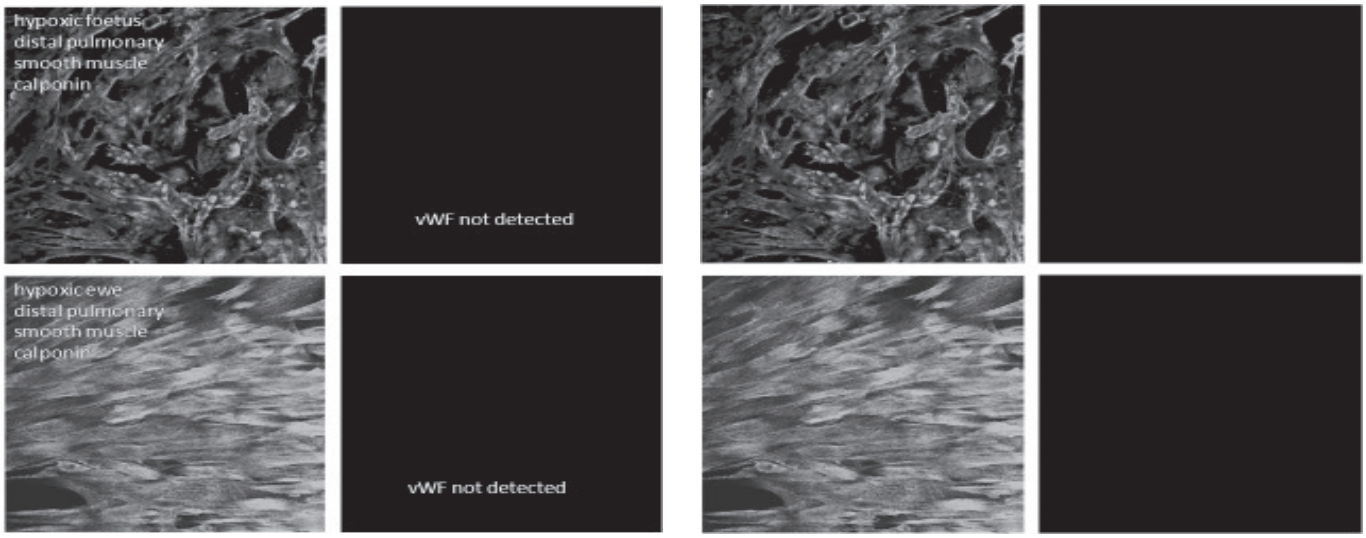


**Figure 4: Fluorescence micrographs of ovine juxta-alveolar smooth muscle cells from chronically hypoxic sheep tagged with alexa-488-labeled monoclonal antibody against alpha smooth muscle actin. Upper panel shows foetal cells, lower panel shows cells of the maternal ewe. Second and fourth columns show failed tagging with alexa-488 labeled anti-von Willebrand factor (endothelial cell marker) in identical monolayers.**

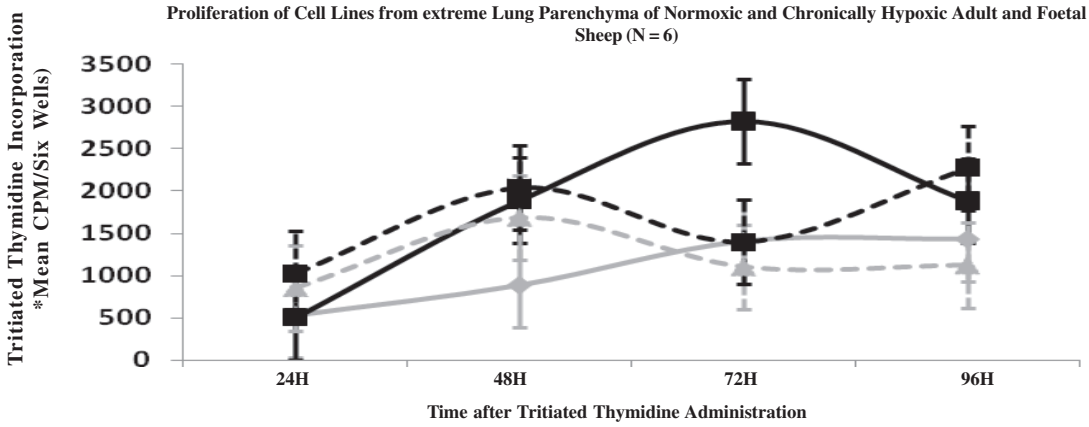


**Figure 5: Growth profiles of primary cultures of smooth muscle cell monolayers obtained by dispase digestion of extreme lung parenchyma (juxta-alveolar smooth muscle cells). normoxic foetus, normoxic ewe, hypoxic foetus an and hypoxic ewe.**

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## DUALITY OF INTEREST

The author declares that there is no duality of interest in this work.

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