Comparison of Inhaled Nitric Oxide Versus Oxygen on Hemodynamics in Patients With Mitral Stenosis and Severe Pulmonary Hypertension After Mitral Valve Surgery

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Pulmonary hypertension represents an important cause of morbidity and mortality in patients with mitral stenosis who undergo cardiac surgery, especially in the postoperative period. The aim of this study was to test the hypothesis that inhaled nitric oxide (iNO) would improve the hemodynamic effects and short-term clinical outcomes of patients with mitral stenosis and severe pulmonary hypertension who undergo cardiac surgery in a randomized, controlled study. Twenty-nine patients (4 men, 25 women; mean age 46 ± 2 years) were randomly allocated to receive iNO (n = 14) or oxygen (n = 15) for 48 hours immediately after surgery. Hemodynamic data, the use of vasoactive drugs, duration of stay, and short-term complications were assessed. No differences in baseline characteristics were observed between the groups. After 24 and 48 hours, patients receiving iNO had a significantly greater increase in cardiac index compared to patients receiving oxygen (p < 0.0001). Pulmonary vascular resistance was also more significantly reduced in patients receiving iNO versus oxygen (−117 dyne/s/cm², 95% confidence interval −34 to −200, vs 40 dyne/s/cm², 95% confidence interval −34 to 100, p = 0.005) at 48 hours. Patients in the iNO group used fewer systemic vasoactive drugs (mean 2.1 ± 0.14 vs 2.6 ± 0.16, p = 0.046) and had a shorter intensive care unit stay (median 2 days, interquartile range 0.25, vs median 3 days, interquartile range 7, p = 0.02). In conclusion, iNO immediately after surgery in patients with mitral stenosis and severe pulmonary hypertension improves hemodynamics and may have short-term clinical benefits. © 2011 Elsevier Inc. All rights reserved. (Am J Cardiol 2011;107:1040–1045)

It has been shown that the pulmonary vasoconstriction present in clinical pulmonary hypertension can be alleviated in the short term using inhaled nitric oxide (iNO) through a mechanism involving vascular smooth muscle relaxation. In humans, the ability of iNO to reduce pulmonary vascular resistance (PVR) while sparing systemic resistance by selective pulmonary vasodilatation has been exploited in patients with right ventricular dysfunction, acute lung injury, and lung transplantation. Specifically in patients with mitral stenosis, in whom chronic left-sided underfilling is prominent, the use of iNO has not been studied thoroughly. Most studies were not randomized or controlled, did not look specifically at clinical outcomes but only focused on the immediate hemodynamic effects of iNO, used iNO during brief periods only, or were restricted to children or women. Fattouch et al compared the use of iNO in mitral stenosis with inhaled prostacyclin and intravenous nitroprusside but did not include a control group not using vasoactive drugs. This is the first randomized controlled study of patients with severe mitral stenosis and pulmonary hypertension using iNO compared to a control group receiving only oxygen. We observed not only the hemodynamic effects of each treatment but also the associated short-term clinical outcomes in these patients.

Methods

The study was approved by the institutional research committee, and all subjects gave written informed consent. Patients were consecutively selected at an outpatient cardiology clinic of a tertiary cardiology referral hospital. Men and women aged ≥18 years were selected if they met all the following inclusion criteria: mitral stenosis with a valve area <1.5 cm²; severe pulmonary hypertension, defined as pulmonary artery systolic pressure (PASP) ≥60 mm Hg; and symptomatic disease with New York Heart Association functional class ≥II. Patients were excluded if they presented with concomitant valvular disease other than mitral stenosis (specifically moderate or important mitral regurgitation as defined by preoperative quantitative echocardiography) or had severe left or right ventricular dysfunction, defined as an ejection fraction <40% by preoperative echocardiography. Nineteen screened patients were
excluded before randomization because of concomitant mitral regurgitation (15 patients) or severe left ventricular dysfunction (3 patients). Central computerized randomization of the treatment assignments was performed. Concealment was interrupted only when the patient was weaned from cardiopulmonary bypass and started to receive the designated therapy.

Before surgery, baseline 2-dimensional echocardiography and Doppler echocardiography were performed using commercially available equipment (Sonos 5500; Philips Medical Systems, Andover, Massachusetts). The ejection fraction, transmural valve gradient, valve area calculated using the pressure half-time method, mitral valve echocardiographic score, and PASP estimated using the modified Bernoulli equation were determined.

Before anesthesia induction in the operating room, a pulmonary artery catheter was placed in the right internal jugular vein or right subclavian vein, and pulmonary capillary wedge pressure, PASP, cardiac output calculated by the pressure half-time method, mitral valve echocardiographic score, and PASP estimated using the modified Bernoulli equation were determined.

Immediately before weaning off cardiopulmonary bypass, patients randomized to the iNO arm had the NO delivery equipment attached to the anesthesia breathing circuit. The surgical team was not aware of the patient randomization allocation until this moment. Inhaled NO was delivered using NO tanks connected to the inspiratory limb of the airflow tubes at a concentration of 10 ppm, a dose with the best cost/benefit ratio according to previous dosing studies. Concentrations of iNO and NO were measured continuously with a dedicated monitoring device (NOxBox; Bedfont Instruments, Rochester, United Kingdom). Patients randomized to the oxygen control group continued to receive standard anesthesia care to maintain oxygen saturation >95%.

All patients were then transferred to a surgical intensive care unit (ICU) and continued the assigned treatment for up to 48 hours, in the ICU or in the ward. The criteria for ICU discharge were prespecified as hemodynamic stability (defined by a mean systemic arterial pressure >65 mm Hg), mechanical ventilation weaning with no signs of respiratory distress (as evaluated by the attending physician, who was blind to the outcomes of the study), urinary output >0.5 ml/kg/hour, and no significant bleeding from surgical sites. No patient was held in the ICU because of the protocol if he or she was considered apt for discharge and achieved the mentioned criteria. After extubation, patients received iNO or oxygen through a facial mask, keeping the aforementioned parameters. In all patients, an airtight sealed nonrebreathing cushioned face mask was used, allowing minimum leakage of either iNO or oxygen. Inhaled NO concentration was measured through a sampling line adjacent to the mask to guarantee a precise reading of the delivered concentration.

The use of any vasoactive drugs was permitted throughout the study and was left to the choice of the attending physician in the ICU, blinded to the outcomes of the study. At 24 and 48 hours after the initiation of iNO or control oxygen, a new reading of the pulmonary artery catheter was carried out, with the determination of the same parameters obtained before surgery. After 48 hours, patients withdrew oxygen therapy unless contraindicated by the assisting physician. Patients receiving iNO were progressively weaned, with total switch to room air or oxygen by mask as needed in 1 hour. All patients were followed during the total hospital stay for the assessment of predefined complications (acute renal insufficiency, infections, need for reintubation, liver failure, and death).

The primary outcomes of this study were the differences at 48 hours compared to the baseline cardiac index and PVR in each treatment group. These primary end points were chosen to allow a more accurate sample size calculation as well as to provide a more relevant link to the clinical effects. Secondary outcomes included clinical variables regarding postoperative complications, total days in the ICU, total hospital stay, and number and dosing of systemic intravenous vasoactive drugs. Complications were defined as acute renal insufficiency (renal output <0.3 ml/kg/hour), need for reintubation, sepsis according to standardized definitions, cardiogenic shock, and need for urgent reoperation.

Data are expressed as mean ± SD, number (percentage), or median (interquartile range).

### Table 1

<table>
<thead>
<tr>
<th>Variable</th>
<th>iNO (n = 14)</th>
<th>Oxygen (n = 15)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>48 ± 11</td>
<td>44 ± 13</td>
<td>0.43</td>
</tr>
<tr>
<td>Women</td>
<td>13 (93%)</td>
<td>12 (80%)</td>
<td>0.60</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>23 ± 4</td>
<td>22 ± 4</td>
<td>0.69</td>
</tr>
<tr>
<td>New York Heart Association class</td>
<td></td>
<td></td>
<td>0.39</td>
</tr>
<tr>
<td>II</td>
<td>3 (21%)</td>
<td>6 (40%)</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>10 (72%)</td>
<td>7 (47%)</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>1 (7%)</td>
<td>2 (13%)</td>
<td></td>
</tr>
<tr>
<td>Atrial fibrillation</td>
<td>8 (57%)</td>
<td>8 (55%)</td>
<td>0.99</td>
</tr>
<tr>
<td>Left ventricular ejection fraction (%)</td>
<td>71 ± 8</td>
<td>70 ± 6</td>
<td>0.48</td>
</tr>
<tr>
<td>Mitral valve echocardiographic score</td>
<td>10.1 ± 1.7</td>
<td>10.6 ± 1.0</td>
<td>0.55</td>
</tr>
<tr>
<td>Mitral valve area (cm²)</td>
<td>0.92 ± 0.18</td>
<td>0.85 ± 0.21</td>
<td>0.35</td>
</tr>
<tr>
<td>Mitral valve mean diastolic gradient (mm Hg)</td>
<td>14.9 ± 3.7</td>
<td>16.4 ± 5.8</td>
<td>0.48</td>
</tr>
<tr>
<td>PASP (echocardiography) (mm Hg)</td>
<td>80 ± 21</td>
<td>80 ± 17</td>
<td>0.71</td>
</tr>
<tr>
<td>Pulmonary capillary wedge pressure (mm Hg)</td>
<td>26.3 ± 10.5</td>
<td>27.9 ± 10.5</td>
<td>0.87</td>
</tr>
<tr>
<td>PASP (pulmonary artery catheterization) (mm Hg)</td>
<td>73 ± 10</td>
<td>73 ± 14</td>
<td>0.99</td>
</tr>
<tr>
<td>Cardiac index (L/min/m²)</td>
<td>2.35 ± 0.6</td>
<td>2.89 ± 0.9</td>
<td>0.10</td>
</tr>
<tr>
<td>PVR (dyne/s/cm³)</td>
<td>341 ± 183</td>
<td>264 ± 133</td>
<td>0.38</td>
</tr>
<tr>
<td>Bypass time (minutes)</td>
<td>88 ± 31</td>
<td>94 ± 34</td>
<td>0.59</td>
</tr>
<tr>
<td>Valve replacement</td>
<td>6 (43%)</td>
<td>3 (20%)</td>
<td>0.25</td>
</tr>
<tr>
<td>Reoperation</td>
<td>4 (29%)</td>
<td>3 (20%)</td>
<td>0.91</td>
</tr>
<tr>
<td>Intensive care unit stay (days)</td>
<td>2.0 (0.25)</td>
<td>3.0 (7.0)</td>
<td>0.02</td>
</tr>
</tbody>
</table>
Figure 1. Values of pulmonary capillary wedge pressure, PASP, cardiac index, and PVR in patients using iNO (black circles) versus oxygen (black squares) preoperatively and at 24 and 48 hours after surgery. There was a significant decrease in PVR at 48 hours in patients using iNO compared to oxygen. *p < 0.005.

Figure 2. Changes compared to baseline of hemodynamic data in patients using oxygen (white bars) and iNO (black bars). Cardiac index increased much more significantly in patients receiving iNO at 24 and 48 hours (p <0.01 for both) compared to preoperative values.
were done using analysis of variance for repeated measures on ranks with Tukey’s and profile tests for time and group differences. Analyses of the differences as well as other comparisons regarding the iNO and control groups were made using unpaired Student’s t tests for continuous variables, Mann-Whitney or chi-square tests for proportions, and Fisher’s exact tests as needed. Sample size was calculated on the basis of an α error of 0.05 and power of 80% to detect a 40% difference in PVR between the 2 groups. We calculated the number of subjects needed for the study to be 30 (15 in each group) on the basis of previous studies. All calculations were done using intention-to-treat analysis and were performed in SAS version 9.1.3 (SAS Institute Inc., Cary, North Carolina). Significance was assumed at a 2-tailed p value <0.05.

Results

Twenty-nine patients (86% women, mean age 46 ± 2 years) were enrolled in the study, with a total evaluation of 48 patients. One patient withdrew consent on the morning of the surgery and was not included in the final analysis. The baseline clinical characteristics of the 2 groups are listed in Table 1. No significant differences between the groups were observed. Patients enrolled were characterized by significant mitral stenosis (mean valve area 0.89 ± 0.04 cm²) with severe pulmonary hypertension (mean PASP 73 ± 3 mm Hg, mean PVR 303 ± 31 dyne/s/cm⁵). Only 1 death occurred; a patient in the oxygen group died 22 days after surgery with sepsis and multiple-organ failure. All randomized patients received the assigned treatment with iNO or oxygen during the full duration of the planned intervention, with no crossovers. Patients in the iNO group were mechanically ventilated for 7.5 ± 8.5 hours, while patients receiving oxygen were ventilated for 15.1 ± 21.8 hours (p = 0.23). No patient developed significant mitral valve regurgitation as assessed by echocardiography 7 days after surgery (data not shown).

Changes in hemodynamic parameters after surgery are presented in Figures 1 and 2. After 24 and 48 hours, the 2 groups had significant reductions in pulmonary capillary wedge pressure, with no differences between patients receiving iNO or oxygen. Significant decreases also occurred in PASP at 24 and 48 hours compared to that at baseline in the 2 groups, with no differences between them. However, the cardiac index increased significantly in patients receiving iNO at 24 and 48 hours (p <0.0001 for both). Moreover, although the cardiac index increased significantly compared to that at baseline in the 2 groups after 24 hours, this increase was sustained at 48 hours only in patients who received iNO, with a mean increase of 1.58 L/min/m² (95% confidence interval [CI] 1.0 to 2.16, p <0.0001) versus 0.4 L/min/m² (95% CI 0.01 to 0.82, p = 0.06) in patients receiving oxygen. PVR changes compared to those at baseline were also observed only in the group with iNO at 24 hours (−103 dyne/s/cm², 95% CI −14 to −192, p = 0.04) and 48 hours (−117 dyne/s/cm², 95% CI −34 to −200, p = 0.02), with significant differences between the 2 groups at 48 hours (p = 0.005). Partial pressure of oxygen was not significantly different between the groups at baseline or at 24 and 48 hours (baseline iNO group 92 ± 7 vs 93 ± 9 mm Hg in the oxygen group, p = 0.90; 24 hours 190 ± 72 vs 221 ± 97 mm Hg, p = 0.40; 48 hours 158 ± 67 vs 193 ± 102 mm Hg, p = 0.50). Although we did not invasively monitor the hemodynamic status of patients after weaning of iNO or oxygen, no patient in the study experienced any clinically relevant hemodynamic symptoms after withdrawal of either therapy.

Patients who received iNO had significantly shorter ICU stays compared to patients who received oxygen (Table 1). However, total hospital stay was similar in the 2 groups (median 10 days, interquartile range 6.0 in iNO group vs median 13 days, interquartile range 16.25 in the oxygen group, p = 0.14).

The use of concurrent vasoactive drugs might have interfered with the results regarding the previous hemodynamic measurements. Table 2 lists systemic intravenous vasoactive drugs used in the 2 groups during ICU and hospital stay. Although no significant differences were observed regarding the percentage of patients using each drug or the maximum dose used in each patient, the mean number of systemic vasoactive drugs used during hospital stay was significantly smaller in the iNO group (2.1 ± 0.14) compared to that in patients receiving only oxygen (2.6 ± 0.16) (p = 0.046). There were a nonsignificant smaller proportion of patients receiving nitroprusside and milrinone in the iNO group compared to controls. Other drugs known to significantly affect pulmonary pressure were not used by any patient in our study either during ICU or ward stay.

The percentage of patients with any of the predefined complications was similar in the 2 groups (9 of 15 [60%] in the oxygen group vs 4 of 14 [29%] in the iNO group, p = 0.14). According to the definitions of complications previously mentioned, 3 patients in the iNO group needed urgent reoperation due to bleeding complications (1 for cardiac tamponade and 2 for >500 ml of sanguineous drainage in the first hour postoperatively), and 1 patient developed sepsis. In the oxygen group, 2 patients needed reintubation, 2 patients underwent urgent reoperation (1 for cardiac tam-
ponade and 1 for high blood drainage), 1 patient developed acute renal failure, and 4 patients were diagnosed with sepsis. Although we did not find any significant differences between the groups regarding complications, we could determine that patients who presented with complications after surgery had significantly fewer change in PVR values compared to patients with no complications at 24 and 48 hours. The mean change in PVR in patients with complications was $4 \pm 42 \text{ dyne/s/cm}^2$ compared to $-124 \pm 34 \text{ dyne/s/cm}^2$ in patients with no complications at 24 hours ($p = 0.02$) and $-31 \pm 41$ and $-98 \pm 37 \text{ dyne/s/cm}^2$, respectively, at 48 hours ($p = 0.03$).

**Discussion**

We have demonstrated that treatment with iNO immediately after surgery in patients with significant mitral stenosis and severe pulmonary hypertension is associated with increased cardiac output and reduced PVR. This in turn translated into shorter ICU stays and less need for systemic vasoactive drug use.

Different therapies for the postoperative management of pulmonary hypertension are available and include intravenous nitroprusside, inhaled prostacyclin, and intravenous phosphodiesterase inhibitors. In contrast with these therapies, iNO is rapidly inactivated through a hemoglobin-mediated process while still in the pulmonary circulation, and established safety profile even up to 40 ppm. In our patients, also not statistically significant, there was a smaller proportion of patients in the iNO group receiving nitroprusside as well as milrinone, and no differences were found in the use of other systemic vasoactive drugs. This reinforces the benefits of iNO use, because these patients exhibited a significant decrease in PVR while using, at most, the same doses of vasoactive drugs.

In our study, we chose to use a dose of 10 ppm of iNO to further minimize any toxic risks associated with the drug and due to previous dose-response studies showing no benefits of increased doses. The 34% reduction in PVR seen in patients using iNO is in accordance with previous reports indicating reductions of PVR up to 27% to 45% with iNO, with more significant reductions being obtained with 20 than at 10 ppm. We also observed an increase in cardiac output after surgery in the 2 groups, but the increase with iNO was significantly greater and sustained. This finding has also been reported by others but was not reproduced in all iNO studies. This apparent discrepancy can be attributed to differences in preoperative cardiac function or hemodynamic conditions, all of which might influence the final cardiac output observed with the treatment. In fact, in the presence of ventricular dysfunction, an increase in left atrial end-diastolic pressure with increased pulmonary blood flow with iNO may lead to pulmonary edema and reduced cardiac output. It is also interesting to point out that despite the reductions in pulmonary hypertension observed in the study, the values of the mean PASP were still elevated in the 2 groups. Part of this is due to the vast and complex pathophysiology of pulmonary hypertension, in which a variety of arterial abnormalities play a role at different stages in the same patient. Therefore, acute interventions in the postoperative setting may revert only part of the functional and structural changes represented by intimal hyperplasia, medial hypertrophy, adventitial proliferation, thrombosis, and inflammation.

The increase in cardiac output and reduced PVR observed in the iNO group in this study was associated with an improvement in several clinical parameters that may result in improved management of these patients after surgery. First, the total ICU stay was reduced using iNO, a finding also reported by others recently. We also found that the use of iNO permitted a more judicious use of systemic vasoactive drugs, with a better hemodynamic profile in these patients despite the use of a reduced number of these agents. Interestingly, a trend was noted toward fewer complications in patients receiving iNO compared to controls, with a shorter total hospital stay. Patients with no complications had significantly higher reductions in PVR postoperatively than did patients presenting with any complications. This may reflect the baseline reversibility of pulmonary hypertension in these patients, but the role of iNO in this reduction cannot be disregarded. Moreover, patients enrolled in this study represented a very high risk population, with a mean PASP of $73 \pm 3 \text{ mm Hg}$, much higher than the 39 to 61 mm Hg reported in other studies. Therefore, any degree of reduction in PVR that would mitigate complications in these patients would be desirable.

Limitations of this study include the hemodynamic readings at 24 and 48 hours in the ICU setting, while the patients were taking other vasoactive drugs that might have confounded our results, although no significant differences were observed in terms of frequency and maximum doses of these drugs in the 2 groups. The use of right ventricular parameters as outcomes of the study would also have enhanced our results, but we believe that other variables studied already give independent and prognostic information regarding the right-sided cardiac chambers to allow the conclusions presented here. Last, although it could be desirable to blind the effective treatment, because we needed to monitor the values of NO for safety reasons, this approach would not seem possible.

Valvular Heart Disease/Nitric Oxide in Mitral Stenosis Surgery


Mother to Child Transfer of IgG and IgA Antibodies Against Dermatophagoides pteronyssinus

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Received 6 June 2011; Accepted in revised form 16 August 2011

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Abstract

There is strong evidence from animal models that placental and/or breast milk-mediated transfer of maternal allergen-specific IgG prevents allergic immune responses in the progeny. Both human and animal data also point to IgA as having an important regulatory role. In contrast, little is known about maternal transfer of IgG and IgA specific for respiratory allergens in humans. Dermatophagoides pteronyssinus (Der p) is an indoor allergen that is a major cause of asthma worldwide. We analysed maternal to child Der p-specific IgG and IgA transfer in a cohort of 77 paired maternal and child samples. We found Der p-specific IgG and its IgG1, IgG2 and IgG4 subclasses in all cord blood samples. Except for IgG1, cord levels were higher in newborns from atopic mothers (n = 29) compared to non-atopic mothers (n = 48). Der p-specific IgA was found in all colostrum samples and levels were independent of maternal atopic status. Notably, anti-Der p IgG was also found in colostrum and levels were higher in atopic mothers. We believe that our work is a critical first step in the identification of early factors that may impact asthma development and should guide the development of clinical studies that assess whether Der p-specific IgG and IgA protect children from allergy as demonstrated in animal models.

Introduction

Atopic asthma affects millions of children worldwide [1]. Pathogenesis of allergic disease results from complex interactions between genetic and environmental factors such as pollution, tobacco and microbial exposure including microbiota of the gastrointestinal tract. In most cases, symptoms of allergic asthma manifest in childhood, and the immunological changes leading to atopy can occur very early in life and even during gestation [2]. Thus, identifying early factors that predispose to asthma development may help to improve primary prevention.

During pregnancy, mothers transfer to the foetus immunoglobulins (Ig) that recognize antigens to which she has been exposed [3]. IgG is the main Ig isotype transferred across the placental barrier [3–5], and its subclasses are ordered according to their relative serum levels: IgG1 > IgG2 > IgG3 > IgG4. IgG1 and IgG3 are generally induced in response to protein antigens, IgG2 is associated with polysaccharide antigens, and IgG4 class switching is stimulated by TH2-type cytokines [6].

After delivery, Ig can be transferred by breastfeeding as it is the most abundant Ig found in human milk [7]. Most studies in humans have focused on placental transfer of IgG or milk transfer of IgA molecules specific for microbial antigens and have demonstrated their role in infectious disease prevention [7, 8]. There is also some evidence from animal models that transferred maternal Ig could exert a regulatory role in their progeny. Experimental data in rodents indicate that maternal allergen-specific IgG transferred by placenta and/or breastfeeding prevents allergic sensitization in the progeny [2, 9–16], and animal and human studies indicate that IgA can exert an immunoregulatory role [17–20]. In humans, only a few studies have demonstrated the presence of IgG [21, 22] or IgA [23–26] specific for food and respiratory antigens in cord blood or breast...
milk, respectively. To date, no study has demonstrated the transfer of IgG specific for respiratory allergens by breast milk.

In this study, we investigated whether mothers can provide to their children antibodies specific for Dermatophagoides pteronyssinus (Der p), a major allergen in house dust and one of the most frequently implicated respiratory allergens in allergic asthma [27–30]. In particular, we assessed whether anti-Der p antibodies were detected in cord blood and/or colostrum and whether maternal atopic status had any influence on the amount of antibody.

Methods

Study design. A total of 77 healthy mothers and their newborns were selected at Maternidade de Campinas Hospital in Campinas, São Paulo, Brazil, between February and July 2006. The selection criteria included mothers giving birth to healthy, full-term and adequate-for-gestational-age-weight infants. Demographic data and details about the antenatal care of the mothers were obtained from their medical records and a directed questionnaire. The information included maternal age, parity, medications during pregnancy and atopic status (e.g. atopic rhinitis or asthma) established by a typical clinical history. Total and Der p-specific IgE were assayed in blood samples from all mothers. Inclusion criteria for atopic mothers were clinical manifestations of rhinitis, asthma or atopic dermatitis and anti-Der p IgE concentration ≥3.5 KU/l \( (n = 29) \). A group of non-atopic healthy mothers (anti-Der p IgE concentration ≤0.3 KU/l and absence of atopic symptoms) was included in the study as a control group \( (n = 48) \). Exclusion criteria for enrolment of all mothers were hypertension, diabetes, infections, immunodeficiency, and those who had received corticosteroids, transfusion of blood-derived products or other drugs related to chronic diseases during pregnancy. The study was approved by the University of São Paulo Institute of Biomedical Sciences Ethics Committee in accordance with the Brazilian Ministry of Health Resolution 96/1996 and the Helsinki Declaration.

Serum and colostrum samples. After mothers signed the informed consent, cord blood was collected from large veins on the foetal side of the placenta immediately after delivery. Maternal peripheral venous blood and colostrum samples were collected within 48 h after delivery. Approximately 5 ml of colostrum was collected manually and, on the same day, centrifuged for 30 min at 160 g at 4 C. The top layer of fat and the pellet were discarded, and the intermediate fluid fraction was aliquoted and stored at −80 C until analysed. Serum was separated from maternal and cord blood and stored at −80 C until assayed.

Total and Der p-specific IgE quantification. Total and anti-Der p IgE antibodies from maternal serum samples were analysed by chemiluminescent immunoenzyme assay (ADVIA Centaur® and Cap System Pharmacia®, respectively), according to manufacturer’s recommendations [31].

In the Cap System Pharmacia® assay, the specific IgE concentration is expressed in KU/l; values ≥3.5 KU/l were considered positive for specific IgE. In the ADVIA Centaur® assay, total IgE concentration is expressed in IU/ml, with a detection level of 1.5 IU/ml.

Total IgA quantification. Total IgA was measured in colostrum samples by enzyme-linked immunosorbent assays (ELISA), as described [32] with modifications. Briefly, colostrum samples were diluted 1:10,000 in duplicate and incubated for 2 h in anti-human IgA (I-0884; Sigma, St. Louis, MO, USA) coated plates. As a standard, we used IgA purified from human colostrums (I-2636; Sigma), and as secondary antibody, peroxidase-conjugated anti-human IgA (A0295; Sigma) diluted 1:6000 (1 h 30 min) was used. Ortho-phenylenediamine (OPD) was used as the chromogenic substrate, and IgA concentration was expressed as mg/ml.

Anti-Der p IgG and IgA quantification. Microplates (Costar, Cambridge, MA, USA) were coated overnight at 4 C with 5 μg/ml of Der p extract from IPI-ASAAC, São Paulo, BR, or with Der p extract from Greer Laboratories, Lenoir, NC, in phosphate-buffered saline (PBS). Both Der p preparations gave similar results. Plates were then saturated with 5% non-fat dry milk in PBS–Tween 0.1% for 1 h at room temperature. Samples and secondary antibodies were added as described below and bound antibodies were revealed by the addition of a solution containing 0.4 mg/ml OPD and 0.01% H2O2 in 0.1 M phosphate–citrate buffer (pH 5.0). After 30 min of incubation, the reaction was stopped with 50 μl of 2.5 N H2SO4. Plates were washed with PBS–Tween 0.1% between each step. Optical absorbance at 492 nm was measured by a microplate reader (Labsystems Multiskan MS, Farnborough, Hampshire, UK).

For Ig detection, sample dilution and secondary antibodies were prepared as follows.

Serum anti-Der p IgG: Maternal and cord serum were added in duplicate at a dilution of 1:100 followed by twofold serial dilutions and incubated at 37 C for 2 h. HRP-conjugated anti-human IgG (A8419; Sigma) at a dilution of 1:400 was used as secondary antibody and incubated at 37 C for 2 h. Results were expressed as arbitrary units (AU)/ml obtained by comparison of optical density (OD) values of a serum pool (collected from 24 mothers with anti-Der p IgE concentration 217.5 KU/l) and defined to contain 1000 AU/ml of anti-Der p IgG.

Serum anti-Der p IgG subclasses: Paired maternal and cord serum samples were added in duplicate at dilutions of 1:5 (IgG1), 1:2 (IgG2) and 1:2 (IgG4), followed by twofold serial dilutions, and incubated for 1.5 h on Der p-coated plates. As secondary antibody, biotinylated anti-human IgG1 (555869; BD Pharmingen, San Diego, CA, USA),
IgG2 (555874; BD Pharmingen) and IgG4 (555882; BD Pharmingen) were used at dilutions of 1:500, 1:1000 and 1:100, respectively, and incubated for 1.5 h. This step was followed by incubation with streptavidin-HRP (554066; BD Pharmingen) diluted 1:500, 1:1000 and 1:500, respectively, for 1.5 h. Concentrations were expressed as arbitrary units (AU/ml) as described previously.

Colostrum anti-Der p IgA: Colostrum samples in duplicate were diluted 1:100 followed by two steps of twofold serial dilutions and incubated at 37 °C for 2 h on purified Der p-coated plates. As secondary antibody, we used peroxidase-conjugated anti-human IgA (A0295; Sigma) diluted 1:6000 and incubated 1.5 h at 37 °C. The results were expressed as arbitrary units (AU/ml) obtained by comparison with a colostrum pool (collected from 24 mothers with anti-Der p IgE concentration ≥17.5 KU/l) and defined to contain 1000 AU/ml of colostrum anti-Der p IgA.

Colostrum anti-Der p IgG: Colostrum anti-Der p IgG quantification was performed as described for colostrum anti-Der p IgA with some modifications: colostrum samples were diluted 1:2 and incubated at 37 °C for 2 h on purified Der p-coated plates. As secondary antibody, we used anti-human biotinylated IgG (555785; BD Pharmingen) followed by streptavidin-HRP (554066; BD Pharmingen), both diluted 1:500 and incubated for 1.5 h at 37 °C. OPD was used as the chromogenic substrate, and concentrations were expressed as arbitrary units (AU/ml) obtained by comparison with a colostrum pool as described previously.

Statistical analyses. Statistical analyses were performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA). Dots represent individual data points, and horizontal lines, the medians of each group. Mann–Whitney test was used to determine statistical differences because the D’Agostino–Pearson normality test was not passed. Kruskal–Wallis test was performed to compare more than two groups. When significant differences were found, a Mann–Whitney test was performed to determine which groups differed. Correlation coefficients of antibody levels in maternal serum versus colostrum or cord blood were determined using Spearman’s tests. Two-tailed P-values <0.05 were considered statistically significant and graphically represented as *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.

Results

Characteristics of the study population

The study population was divided into two groups according to maternal atopic status defined by the presence of anti-Der p IgE ≥ 3.5 KU/l and associated allergic symptoms. The characteristics of the two groups are summarized in Table 1. Total and anti-Der p IgG concentrations in blood samples from atopic mothers were significantly higher than non-atopic mothers (Table 1). Maternal age, infant weight and height, and male-to-female ratios were similar between the two groups (Table 1).

Der p-specific IgG1, IgG2 and IgG4 in cord blood and maternal serum

Anti-Der p IgG2 and IgG4 concentrations were significantly higher in cord blood of neonates from atopic mothers compared to neonates from non-atopic mothers (Fig. 1A and Table 2). In addition, neonatal anti-Der p IgG correlated with anti-Der p IgE levels in maternal blood (data not shown; Spearman r = 0.2, P = 0.006). Similarly to their children, atopic mothers showed higher concentration of anti-Der p IgG compared to non-atopic mothers (Fig. 1A and Table 2), and Der p-specific IgG in maternal blood correlated with anti-Der p IgE levels (Spearman r = 0.2, P = 0.009). Anti-Der p IgG levels in cord blood correlated strongly with the maternal concentration for both atopic and non-atopic groups (Fig. 1B). The ratio of cord blood to maternal blood anti-Der p IgG levels was not affected by maternal antibody concentration (Fig. 1C).

Der p-specific IgG1, IgG2 and IgG4 in cord blood and maternal serum

Anti-Der p IgG2 and IgG4 concentrations were significantly higher in cord blood of neonates of atopic mothers compared to non-atopic mothers (Fig. 2B,C and Table 2),

Table 1 Characteristics of the study population.

<table>
<thead>
<tr>
<th></th>
<th>Atopic (n = 28)</th>
<th>Non-atopic (n = 48)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mother</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total IgE (UI/ml)</td>
<td>263.2 (17.3–3910)****</td>
<td>16.45 (1.5–1543)</td>
</tr>
<tr>
<td>Der p-specific IgE (KU/ml)</td>
<td>20.7 (3.4–100)****</td>
<td>&lt;0.35 (&lt;0.35–0.35)</td>
</tr>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gestation, % (number)</td>
<td>26.5 (16–40)</td>
<td>26.5 (13–37)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Newborn</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>3.2 (2.3–4.2)</td>
<td>3.3 (2.4–4.5)</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>48.6 (45–53)</td>
<td>49.1 (44.5–52.8)</td>
</tr>
<tr>
<td>Sex (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>52</td>
<td>42</td>
</tr>
<tr>
<td>Female</td>
<td>48</td>
<td>58</td>
</tr>
</tbody>
</table>

Data represent median values and range (shown in brackets). Results obtained in atopic and non-atopic groups were compared, and statistical difference was calculated by Mann–Whitney test.

****Statistically significant values.
while the anti-Der p IgG1 concentration was equivalent in both groups (Fig. 2A and Table 2). Further, cord blood anti-Der p IgG2 and IgG4, but not IgG1, correlated with maternal anti-Der p IgE concentrations (Spearman $r = 0.2$, $P = 0.03$ and $r = 0.5$, $P < 0.0001$ for IgG2 and IgG4, respectively). As observed in the neonates, maternal blood IgG2 and IgG4 levels were higher in the serum of atopic mothers compared to non-atopic, while IgG1 levels were similar in both groups (Fig. 2A–C), and Der p-specific IgG subclasses in maternal blood correlated with anti-Der p IgE levels with the exception of IgG1 (data not shown; Spearman $r = 0.2$, $P = 0.03$ and $r = 0.5$, $P < 0.0001$ for IgG2 and IgG4, respectively).

Cord blood anti-Der p IgG1, IgG2 and IgG4 correlated strongly with respective maternal levels in both groups (Fig. 2D–F), and the ratio of cord blood to maternal blood antibody levels decreased at high maternal antibody concentration (Fig. 2G–I). We also found that the ratio of cord blood to maternal serum anti-Der p IgG1 was higher than for the other IgG subclasses in both groups (Table 2).

Der p-specific IgA and IgG in colostrum

Total and anti-Der p IgA were detected in all colostrum samples without significant differences between atopic and non-atopic mothers (Fig. 3A). For both groups, a positive correlation was found between total and anti-Der p IgA concentrations in colostrum (Fig. 3B). We did not find any correlation between anti-Der p IgA levels in colostrum and anti-Der p IgG or IgE levels in maternal blood, as expected, because most of the IgA found in colostrum is produced peripherally, in mammary tissue.

In addition to anti-Der p IgA, we found anti-Der p IgG in all colostrum samples (Fig. 4 and Table 2). Anti-Der p IgG concentrations in colostrum were higher in atopic mothers (Fig. 4A) and correlated with maternal anti-Der p IgE concentrations (Spearman $r = 0.3$; $P = 0.002$). Colostrum anti-Der p IgG concentrations correlated with maternal blood anti-Der p IgG in the non-atopic group but not in the atopic group (Fig. 4B).

Discussion

This study demonstrates the presence of Der p-specific IgG in all cord blood samples as well as Der p-specific IgA and IgG in all colostrum samples. Others have previously shown the presence of IgG antibodies specific for respiratory antigens from birch pollen (Bet v 1), cat (Fel d 1) and Dermatophagoides farinae (Der f 1) in cord blood samples [22, 33, 34]. In those studies, not all samples were positive, which probably reflects differences in immunogenicity of the allergen tested and in the maternal exposure to the allergens. In this case, Der p is an indoor allergen that is widely distributed in the humid regions of the world [27–30].

The analysis of IgG subclass concentrations in maternal and cord blood demonstrates that cord blood concentrations of anti-Der p IgG, IgG1, IgG2 and IgG4
correlated strongly with respective maternal values. We also found that both maternal serum and cord blood anti-Der p IgG, IgG2 and IgG4 correlated with maternal IgE levels, and we found higher levels of IgG, IgG2 and IgG4 in cord blood of neonates from atopic mothers compared to non-atopic mothers. Such correlation was not found for anti-Der p IgG1, and concentrations of IgG1 were equivalent in both groups. In addition, as previously described by others [33], we detected anti-Der p IgG and subclasses in maternal serum and cord blood in the absence of maternal Der p-specific IgE. In addition to the presence or absence of atopy, differences in maternal exposure to Der p could also be responsible for differences in IgG levels in maternal blood, colostrum and cord blood. Although we did not measure Der p levels in subjects’ homes, we did not favour this hypothesis because all subjects live in a region where Der p is found uniformly in very high concentration [35].

The source of the Der p-specific IgG found in cord blood might be of foetal origin as a result of in utero sensitization or might be of maternal origin as a result of maternal transfer across the placenta. Many studies have reported that allergen-specific IgE detected in cord blood is synthesized in utero and can be a marker of risk of atopic disease development in children [36–38]. However, this concept was recently challenged by Bonnellykke et al. [4, 5]. Comparison of allergen-specific IgE in maternal and cord blood indicated that specific IgE in cord blood completely matched specific IgE in maternal blood with respect to allergen specificity, level of specific IgE and ratio of total IgE to specific IgE. In addition, corresponding specific IgE was not found in infant blood at 6 months of age. These observations let the authors conclude that the presence of cord blood IgE was, in the majority of cases, a result of maternal transfer. Our results showed a strong correlation between cord blood anti-Der p IgG, IgG1, IgG2 and IgG4 and respective maternal levels. Although we do not have data on IgG levels in children at 6 months of age, our data suggest a maternal transfer of anti-Der p IgG subclasses across placenta.

In addition, the decreased ratio of cord blood to maternal levels of these antibodies at high maternal concentrations suggests a saturable receptor-mediated transfer. Notably, the syncytiotrophoblast expresses a neonatal Fc receptor (FcRn) that is essential for IgG transfer [3, 39] and is saturable [40]. This receptor has a higher affinity for IgG1, compared to other IgG subclasses, which may explain the more efficient in utero transfer of Der p-specific IgG1, compared to other subclasses [3], as also shown here.

Several studies in rodents have reported that maternal allergen-specific IgG inhibits allergic responses in the offspring [2, 9–16]. Proposed mechanisms of protection by maternal IgG include the following: (1) IgG binding to allergen, leading to allergenic determinant masking and clearance of the immune complexes by phagocytosis, (2) IgG blockade of IgE binding to allergen and hence inhibition of mast cell degranulation and (3) interactions with inhibitory receptor FcγRIIB on neonatal B lymphocytes or dendritic cells [2, 41]. More recently, protection from allergic airway disease by antigen transfer through breast milk was shown to be more stronger and of longer

### Table 2 Der p-specific IgG levels in maternal serum and colostrum and neonate umbilical cord serum.

<table>
<thead>
<tr>
<th></th>
<th>Maternal serum</th>
<th>Umbilical cord serum</th>
<th>Colostrum</th>
<th>Cord/maternal serum ratio (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Atopic (n = 29)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-Der p IgG</td>
<td>1266 (130–3321)</td>
<td>1121 (84–3704)</td>
<td>352 (17.8–10807)</td>
<td>98.72 (52.5–182.1)</td>
</tr>
<tr>
<td>Anti-Der p IgG1</td>
<td>135.5 (10.9–1205)</td>
<td>213.1 (56.2–1902)</td>
<td>–</td>
<td>177.7 (78.1–462.9)</td>
</tr>
<tr>
<td>Anti-Der p IgG2</td>
<td>96 (4.8–1344)</td>
<td>65.8 (1.5–1162)</td>
<td>–</td>
<td>89.9 (31.2–189.1)</td>
</tr>
<tr>
<td>Anti-Der p IgG4</td>
<td>44.6 (0.5–5357)</td>
<td>29.4 (0.0–4498)</td>
<td>–</td>
<td>88.7 (0.0–222)</td>
</tr>
<tr>
<td><strong>Non-atopic (n = 48)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-Der p IgG</td>
<td>656.4 (69–3585)</td>
<td>606.1 (67–3296)</td>
<td>115.6 (3.5–1061.2)</td>
<td>94.35 (62.86–187)</td>
</tr>
<tr>
<td>Anti-Der p IgG1</td>
<td>96.2 (0–405)</td>
<td>144.7 (17.6–1366)</td>
<td>–</td>
<td>170.5 (66.5–1398)</td>
</tr>
<tr>
<td>Anti-Der p IgG2</td>
<td>37.6 (0.8–1219)</td>
<td>33 (0.6–1393)</td>
<td>–</td>
<td>82.1 (17.39–8441)</td>
</tr>
<tr>
<td>Anti-Der p IgG4</td>
<td>4.9 (0.0–42.3)</td>
<td>3.1 (0.0–48.5)</td>
<td>–</td>
<td>87.8 (5.4–1317)</td>
</tr>
</tbody>
</table>

**, not performed.
Anti-Der p IgG levels are expressed in AU/ml. Data represent median values and ranges (shown in bracket). Cord/maternal serum ratios represent the median of the ratio calculated for each pair of cord/maternal samples. For both atopic and non-atopic group, we compared IgG1, IgG2 and IgG4 cord/maternal serum ratio.

***Statistically significant values.
duration when maternal allergen-specific IgG is present in breast milk. The authors attributed the increased protection to the formation of allergen–IgG immune complexes that are easily transferred across the neonatal gut barrier compared to uncomplexed antigen and display tolerogenic properties [42].

Human studies also suggest an immunoregulatory role for in utero transfer of maternal IgG. A study by Glovsky et al. [43] analysed the effect of specific immunotherapy during pregnancy on allergic sensitization in children. Their data suggested that blocking antibodies induced by immunotherapy were transferred across the placenta and were responsible for decreased allergic sensitization in their children. Jenmalm and Björkstén [21] found that high concentration of IgG directed to inhaled allergens in cord blood was associated with reduced atopy in children. Another study showed a transient protective effect of placental transfer of maternal antibodies on allergic immune response [22]. The current study demonstrated a higher concentration of specific IgG4 and, to a lesser extent, of IgG2 in cord blood of neonates from atopic mothers compared to non-atopic mothers. Although we cannot conclude that these IgG subclasses exert an immunoregulatory role, a protective effect has previously been reported for IgG4 [44–46].

After delivery, breastfeeding maintains a strong interaction between the mother and her infant and in particular allows the continued transfer of maternal Ig. Secretory IgA is the predominant class of Ig found in human breast milk. This class of non-inflammatory Ig inhibits microbial colonization through decreased adherence of bacteria and viruses to mucosal surfaces and thereby protects against gut and respiratory infections in breastfed children [7]. IgA can also trap food antigens, leading to immune exclusion of dietary antigens by favouring degradation by pancreatic enzymes [47]. In addition to
immune exclusion, IgA can exert immunoregulatory effects [17–20]. The epidemiological evidence of food allergy prevention by IgA [48–51] might be explained by these two mechanisms. As the majority of inhaled antigens reach the gut [52], the presence of milk-borne Der p-specific IgA may then protect the newborn from respiratory allergens as proposed for food allergens. Notably, we found anti-Der p IgA in all colostrum samples tested. The range of values was broad, and we did not observe significant differences in antibody concentrations between atopic and non-atopic mothers. One previous study assessed the presence of IgA to cat allergen in human breast milk from atopic and non-atopic mothers. This study also found a similar concentration of IgA in both groups [26]. The absence of an effect of atopy on IgA levels could be explained by the fact that IgA class switching depends mainly on the presence of TGF-β [53]. In fact, we found similar levels of TGF-β in colostrum of atopic and non-atopic mothers, and we observed that both total IgA and Der p-specific IgA levels correlated with TGF-β levels in colostrum (Figure S1).

In addition to IgA specific for respiratory allergen, our study demonstrated, for the first time, the presence of Der p-specific IgG in colostrum. Der p-specific IgG concentrations were higher in colostrum from atopic mothers compared to non-atopic mothers, and colostrum levels correlated with maternal IgE serum levels. It is worth noting that colostrum Der p-specific IgG concentration correlated with maternal serum IgG levels in the non-atopic but not in the atopic group. IgG in colostrum could come from maternal serum, as supported by the observation that intravenous administration of Ig to immunodeficient mothers results in the presence of Ig in breast milk [54]. In addition, IgG maybe synthesized locally in the mammary gland. The latter mechanism may operate in the atopic group because there was no correlation between maternal serum and colostrum Der p-specific IgG levels in that group. Studies in rodents suggest that, as in the placenta, FcRn can be involved in IgG transfer across mammary gland epithelium [55]. Notably, in contrast to IgA that stays in the gut lumen, anti-Der p IgG can then be transferred to the neonate by FcRn expressed in the human proximal intestine [39, 56]. This continuous supply of IgG after delivery may be sufficient to maintain systemic levels in the neonate and allow protection from asthma when in utero-transferred
antibodies have been degraded. In support of this hypothesis, a meta-analysis of prospective studies and a multidisciplinary review of studies performed between 1966 and 2000 concluded that breastfeeding protection from asthma was higher in the subgroup of children with a positive family history of asthma or atopy compared with children with no parental history of atopy [57, 58]. In the light of experimental data obtained in animal models, our work suggests that the higher concentration of Der p-specific IgG in colostrum from atopic mothers may contribute to the better protection afforded upon breastfeeding by atopic mothers.

Conclusions

Our study indicates that Der p-specific IgG can be found in both cord blood and colostrum and identifies maternal atopy as a critical factor for increased levels of allergen-specific IgG in these compartments. In addition, Der p-specific IgA is present in colostrum. Clinical studies will be necessary to assess whether Der p-specific IgG and IgA protect the child from allergy as demonstrated in animal studies. In view of the increasing evidence from animal models and importance of neonatal prevention of allergy, this study would be a timely and necessary way to elucidate the role of allergen-specific Ig in early life and its effect on allergy development.

Acknowledgment

The authors thank Maternidade de Campinas Hospital, Prof. Maria Notomi Sato (Laboratory of Clinical and Experimental Allergy and Immunology, School of Medicine, University of São Paulo) for supplying us with anti-human IgG antibodies, Dr José Carlos Mori (IP-ASAC, Brasil) for Der p purified extract, nurse Silvana S. Dalgé for her excellent assistance in the colostrum collection, Dr Meri Tulic and Dr Peter Newburger for critical reading of the manuscript, as well as the mothers who kindly agreed to participate in this study. We also acknowledge the State of São Paulo Research Foundation (FAPESP) for financial support: Grant 08/58825-7 to Antonio Condino-Neto, Grants 05/57593-7 and 08/51535-3 to Patricia Macchiaverni.

References

5 Bonnelykke K, Pippert CB, Bisgaard H. Transfer of maternal IgE can be a common cause of increased IgE levels in cord blood. *J Allergy Clin Immunol* 2010;126:657–63.
9 Jarrett EE, Hall E. IgE suppression by maternal IgG. *Immunology* 1983;48:49–58.
25 Rumboto M, Chirido FG, Anon MC, Fossati CA. Detection and characterization of antibodies specific to food antigens (gliadin, ovalbu-
34 Jennmalm MC, Holt PG, Bjorksten B. Maternal influence on IgG subclass antibodies to Bet v 1 during the first 18 months of life as detected with a sensitive ELISA. Int Arch Allergy Immunol 1997;114:175–84.

Supporting Information

Additional supporting information may be found in the online version of this article.

**Figure S1** Colostrum IgA levels are correlated to colostrums TGF-β levels in colostrum. TGF-β levels were determined in colostrum samples by ELISA according to manufacturer instruction (Promega, CAT G 7591). Data obtained in colostrum from atopic and non atopic mothers are compared by Mann–Whitney test (a). TGF-β concentrations obtained in colostrum are correlated with colostrum total IgA (b) and colostrum Der p-specific IgA (c) using Spearman test.

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Amlodipine Reduces Cardiac Iron Overload in Patients with Thalassemia Major: A Pilot Trial

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ABSTRACT

BACKGROUND: Iron chelation therapy in patients with thalassemia major may not prevent iron overload in all organs, especially those in which iron enters cells through specific calcium channels. We designed a controlled pilot study to assess the potential of the calcium channel blocker amlodipine in strengthening the efficacy of iron chelation.

METHODS: Fifteen patients with thalassemia major undergoing chelation therapy were randomized to receive amlodipine added to standard treatment in a 1:2 allocation for 12 months. T2* values for assessment of iron overload in the liver and heart using magnetic resonance imaging were obtained at baseline and at 6 and 12 months.

RESULTS: In the amlodipine-treated group, heart T2* increased significantly in comparison to baseline at 6 and 12 months (21.7 ± 6.7 ms to 28.2 ± 7.9 ms and 28.3 ± 8.0 ms, with P = .007 and .03, respectively), while no differences were observed in the control group (25.1 ± 8.8 ms to 24.7 ± 7.8 ms and 26.2 ± 11.4 ms; P = .99 and 0.95, respectively); significant differences between groups were observed at 6 months (28.2 ± 7.9 ms vs 24.7 ± 7.8 ms in the control group, P = .03). A significant reduction in ferritin levels also was observed in the treated group at 12 months.

CONCLUSIONS: The use of amlodipine in conjunction with standard chelation therapy may suggest a new strategy in preventing and treating iron overload in patients with thalassemia major, especially in organs where iron absorption depends on active uptake by calcium channels like the heart.
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KEYWORDS: Iron overload; Magnetic resonance imaging; Thalassemia

Iron overload in thalassemia major is one of the main prognostic factors in the management of the disease, leading to reduced quality of life and long-term chelation therapy, which is associated with multiple side effects.1 All chelation strategies in thalassemia major have been limited so far to the removal of iron from different organs, but because iron moves in and out of cells through distinct mechanisms, some iron chelators might not be so effective in removing previously stored iron, with the need for higher doses or combination therapy, and worsening of potential side effects.2 Therefore, lowering cellular iron uptake may be a potential complementary treatment to standard iron chelation.

Previous in vitro studies showed that L-type calcium channels provide a major pathway for iron entry into cardiomyocytes3-5 and, in mice, calcium channel blockers reduce myocardial iron overload. Thus, we investigated in a pilot study the use of the calcium channel blocker amlodipine in reducing iron overload in patients with thalassemia major.

METHODS

Patients

The pilot study was designed as an open-label, controlled trial. From a group of 60 patients who had repeatedly undergone a magnetic resonance (MR) examination since
2003, 15 patients were included in the study based on the criteria of at least 7 years of age (for compliance with the MR examination), regular transfusions, and iron overload with no perspective of changing the chelation therapy in the following 6 months. Exclusion criteria were patients with significant left ventricular dysfunction (ejection fraction < 35%), renal insufficiency, advanced atrioventricular conduction disturbances, and formal contraindications to MR examinations. To minimize an impact from changes in chelation during the study, patients chosen had a previous history of good compliance, and it was not planned to alter their current chelation regimen in the short run. Sample size was chosen based on the 45% reduction of myocardial iron observed in studies with mice, considering an initial mean cardiac T2* of 20 ms with a standard deviation of 5 ms, power of 0.8, and an alpha error of 0.05 (PASS 11; NCSS LLC, Kaysville, Utah).³ The study was approved by the local Ethics Committee and all patients provided written informed consent. The study is registered at www.ClinicalTrials.gov as NCT01125254.

**Study Design**

At baseline, patients were assessed for clinical characteristics as well as laboratory data, including ferritin levels. All patients also underwent an MR scan (1.5T Siemens Symphony; Siemens Medical Solutions, Erlangen, Germany) for evaluation of left ventricular volumes and function as well as both heart and liver T2* iron quantification according to previously published standards, using the truncation model.⁶ Heart and liver T2* values were calculated using CMR42 software (Circle Cardiovascular Imaging Inc., Calgary, AB, Canada) in batch and random order by 2 readers (JLF and EFS) with the mean of the 2 readings used. Liver iron concentrations were calculated using previous published data by Wood et al and quoted in dry weight.³ After baseline measurements, patients were randomized to receive amlodipine 5 mg/d for 12 months in a 1:2 allocation. Attending physicians were allowed to change the initial dose if blood pressure was lower than 90 × 60 mm Hg or if symptoms or side effects were important. All patients repeated laboratory and MR evaluation at 6 and 12 months. Although the drug was given open label, the readers of the MR images were blinded to treatment allocation.

**Outcomes and Statistical Analysis**

The primary outcome of the study was the difference in heart T2* values between groups at 12 months. Secondary outcomes included myocardium T2* changes at 6 months as well as serum ferritin and liver iron concentration at both time points. Ferritin, heart T2*, and liver iron concentration were normally distributed, so parametric tests were used. Baseline differences between treatment and control groups were compared using Student’s t test or chi-squared. Changes within the same group were analyzed using analysis of variance for repeated measures with Bonferroni correction for pairwise comparisons. Comparison between groups at different time points was done with analysis of covariance with group as the factor and baseline values as covariate to account for potential differences in the baseline values.

**RESULTS**

Baseline characteristics of patients are shown in the Table. No significant differences were found among the participants, including the type of chelation therapy, although ferritin and hemoglobin levels had a trend to be higher in the control group. One patient in the treatment group died at 10 months of follow-up due to liver failure secondary to advanced cirrhosis already present at baseline. All analysis that included 12-month data did not include this patient. All treated patients used 5 mg/d of amlodipine initially, with one patient having to reduce the dose to 2.5 mg/d due to lower extremities edema. No serious adverse events related to amlodipine were detected. No significant reductions in systemic blood pressure were observed and no significant changes in chelation therapy were made during the trial in the patient or control group (type of medication or dosing).

At 6 and 12 months, heart T2* in the treatment group significantly increased from 21.7 ± 7.2 ms to 28.2 ± 7.9 ms and 28.3 ± 8.0 ms (P = .007 and .03, respectively), with no significant changes in the control group (25.1 ± 8.8 ms to 24.7 ± 7.8 ms and 26.2 ± 11.4 ms; P = .99 and .95, respectively) (Figure). Compared with controls, treated patients at 6 months had significantly higher heart T2* values (P = .03), although this difference was not significant at 12 months (P = .3).

A reduction in ferritin levels also was observed in treated patients compared with controls at 12 months (P = .017). Compared with baseline, ferritin levels for treated patients fell nonsignificantly from 1110 ± 837 ng/mL to 545 ± 198 ng/mL and 453 ± 285 ng/mL at 6 and 12 months (P = .42 and P = .66, respectively). Control patients did not show significant changes from baseline (1602 ± 1084 ng/mL to 1673 ± 1194 ng/mL and 1608 ± 842 ng/mL; P = .97 and .99, respectively).

Liver iron concentration did not change significantly in both groups along the 12 months of follow-up from

**CLINICAL SIGNIFICANCE**

- Iron chelation may not prevent uptake of iron in all organs, especially in those in which iron enters cells through specific ion channels.
- Calcium channel blockade with amlodipine in patients with thalassemia major reduced iron overload in the heart in association with standard chelation.
- Amlodipine might serve as a new complementary treatment to standard chelation regimens in patients with thalassemia major, without the burden of significant costs or side effects.
baseline, and no significant differences were found between groups at 6 or 12 months.

**DISCUSSION**

We showed that calcium channel blockade with amlodipine may represent a novel and complementary treatment for iron overload in patients with thalassemia major. Our results are in accordance with previous data in mice that showed a 50% reduction of myocardial iron levels after a 3-month treatment with verapamil. While an improvement of only approximately 30% in heart T2* was observed after 12 months, this increase is still significant compared with increases in heart T2* of 40%-50% observed with intensive chelation therapy or of 15%-31% with chelation monotherapy, also at 1 year. As expected, liver iron concentrations did not change because iron uptake in the liver is less dependent on calcium channels compared with the myocardium. The decrease in ferritin was quite unexpected; we speculate that other factors, such as a reduction in inflammation, may be responsible for these changes. Another explanation might be the increased extracellular availability of iron for the chelators once these atoms are averted from entering sites blocked by amlodipine, although one might expect a reduction in liver iron concentration as well in this case. Finally, we cannot exclude the possibility that increased urinary iron excretion played a role in the elimination of iron in treated patients, as amlodipine prolongs the opening of divalent metal transporter-1 in the kidneys.

<table>
<thead>
<tr>
<th>Table</th>
<th>Baseline Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Variable</td>
<td>Amlodipine Group (n = 5)</td>
</tr>
<tr>
<td>Age, y (range)</td>
<td>31.2 ± 3.9 (25-35)</td>
</tr>
<tr>
<td>Males, n (%)</td>
<td>3 (60)</td>
</tr>
<tr>
<td>Height (m)</td>
<td>1.68 ± 0.06</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>65.4 ± 15.5</td>
</tr>
<tr>
<td>BSA (m²)</td>
<td>1.73 ± 0.22</td>
</tr>
<tr>
<td>Chelation therapy</td>
<td></td>
</tr>
<tr>
<td>DFO, n (%)</td>
<td>2 (40)</td>
</tr>
<tr>
<td>DFP, n (%)</td>
<td>1 (20)</td>
</tr>
<tr>
<td>DFX, n (%)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>DFO + DFP, n (%)</td>
<td>2 (40)</td>
</tr>
<tr>
<td>Ferritin, ng/mL</td>
<td>1110 ± 837</td>
</tr>
<tr>
<td>Hemoglobin g/dL</td>
<td>9.4 ± 0.65</td>
</tr>
<tr>
<td>Splenectomy (%)</td>
<td>2 (40)</td>
</tr>
<tr>
<td>Hepatitis C (%)</td>
<td>3 (60)</td>
</tr>
<tr>
<td>Heart T2* (ms)</td>
<td>21.7 ± 7.2</td>
</tr>
<tr>
<td>Liver iron concentration (mg/g dry weight)</td>
<td>6.5 ± 4.3</td>
</tr>
<tr>
<td>Left ventricular ejection fraction (%) (range)</td>
<td>66.5 ± 9.2 (55.2-78.0)</td>
</tr>
<tr>
<td>Indexed diastolic left ventricular volume (mL/m²)</td>
<td>83.2 ± 23.2</td>
</tr>
<tr>
<td>Indexed systolic left ventricular volume (mL/m²)</td>
<td>29.1 ± 15.5</td>
</tr>
</tbody>
</table>

BSA = body surface area; DFO = deferoxamine; DFP = deferiprone; DFX = deferasirox.

*Data are presented as means ± SD.

Figure Mean values and 95% confidence interval limits for ferritin, heart T2*, and liver iron concentration at baseline, 6, and 12 months in the control group (triangles, dashed line) and in patients treated with amlodipine (open circles, solid line). *P < .05 comparing groups; †P < .05 comparing changes from baseline at 6 and 12 months.
The choice of amlodipine over other calcium channel blockers resides on its safety profile (including pediatric populations), as well as its negligible effect on inotropism and atrioventricular conduction at normal doses. This could extend the use of the drug in patients with left ventricular dysfunction, a condition in thalassemia major patients where cardiac iron overload is usually more significant.

Our small study had limitations inherent with its exploratory nature. We were unable to assess whether the observed changes in surrogate end points might correspond to clinical benefits or heart function improvement, especially changes in left ventricular ejection fraction. We also did not explore other organs that also may suffer from calcium channel-mediated iron overload. Finally, we decided not to continue with the originally planned cross-over study after 12 months, as the results of the interim analysis suggested the need to proceed to a more robust randomized, blinded study that is currently ongoing (www.ClinicalTrials.gov NCT01395199). The lack of significant differences between groups at 12 months despite the significant findings at 6 months and the differences observed within groups for the amlodipine arm, also point to the need of a larger cohort.

In conclusion, the addition of a calcium channel blocker to standard chelation in patients with thalassemia major may improve the efficacy of heart iron removal and reduce serum levels of ferritin, with further testing for assessment of safety and clinical benefits still needed.

References