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Aquaporine-5 and epithelial sodium channel β-subunit gene expression in gastric aspirates in human term newborns

Fabiola Castorena-Torres [a], **Mario René Alcorta-García** [a,b], **Víctor Javier Lara-Díaz** [a,*]

[a] *Tecnologico de Monterrey, Escuela de Medicina y Ciencias de la Salud, Ave. Ignacio Morones Prieto 3000 Pte, Edificio CITES, Piso 3, Col. Doctores, CP 64710, Monterrey, Nuevo León, Mexico*

[b] *Hospital Regional Materno Infantil de Alta Especialidad, Departamento de Neonatología, Servicios de Salud O.P.D. Nuevo León. Avenida San Rafael No. 450, Col. San Rafael, CP 67140, Guadalupe, Nuevo León, Mexico*

[*] Corresponding author.

E-mail address: lara-diaz.vj@itesm.mx (V.J. Lara-Díaz).

Abstract

Both transient tachypnea of the newborn and neonatal respiratory distress syndrome have been associated with changes in gene expression of aquaporine-5 (AQP5) and the β subunit of the epithelial sodium channel (β-ENaC) in the respiratory epithelium. Gastric aspirate (GA) obtained immediately after birth could represent a new source for gene expression analysis for these respiratory diseases. The aims of this study were to determine the feasibility of estimating AQP5 and β-ENaC gene expression in exfoliated respiratory epithelial cells from the GA of term neonates, and to compare the values with those found in scraped nasal epithelial cells, previously validated as a surrogate for distal lung epithelium in terms of ionic channel activity. The study had a cross-sectional, proof-of-concept design. Immediately after birth, we obtained GA and nasal mucous membrane scrapings from term newborns, in which total RNA and RT-qPCR assays for AQP5 and β-ENaC genes were performed. AQP5 gene expression was greater in GA than in nasal scrapings, and β-ENaC gene expression was at least as great in GA as that obtained in nasal scrapings. Amplification of samples from the two sites was comparable. AQP5 gene

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expression was greater in babies delivered by cesarean section; β-ENaC gene expression was greater in babies delivered vaginally, but only in the nasal samples. Quantitation of the expression of AQP5 and of β-ENaC genes in GA, obtained shortly after birth from term newborns is feasible. If confirmed in preterm neonates, this approach could aid in the differential diagnosis of neonatal respiratory diseases.

Keywords: Medicine, Pediatrics, Physiology

1. Introduction

During intrauterine life in mammals, secretion of ions and water by the respiratory epithelium into the lumen of the airways contributes to the growth and differentiation of the respiratory tree [1]. Around the time of birth, in preparation for normal pulmonary gas exchange, the net extrusion of sodium flow through the epithelial sodium channel is reversed to allow absorption of this ion, thereby changing the electrolyte's composition of the lung fluid. This phenomenon occurs in part as a response to the rise in glucocorticoid and catecholamine's concentrations that is associated with birth at term [2, 3], and in part as a result of the sudden increase in oxygenation that occurs upon exposure to aerial respiration [4], and is discussed in depth elsewhere [1, 2, 3, 4, 5, 6, 7, 8, 9, 10]. Two of the most common respiratory disorders in the human newborn, neonatal respiratory distress syndrome (RDS) and transient tachypnea of the newborn (TTN) have been associated with changes in the respiratory epithelium gene expression of the aquaporine-5 (AQP5) and the β subunit of the epithelial sodium channel (β-ENaC). RDS, which affects mainly premature infants, is characterized by a relative surfactant deficiency superimposed on an inability to adequately absorb fetal lung liquid [1, 11]; a decrease in the expression of β-ENaC has been strongly associated with this entity [7, 8, 9, 10]. TTN is triggered mainly by the delay of fetal lung liquid clearance [5, 7], it is more common in late-preterm and early-term newborns; and has been associated with an increment of the expression levels of AQP5 in tracheal aspirates [5, 7, 8, 9, 10].

The aims of this study were to determine the feasibility of measuring the expression of AQP5 and of β-ENaC genes in exfoliated respiratory epithelial cells contained in the gastric aspirate (GA), obtained immediately after birth from term newborns, and to compare the values with those found in scraped nasal epithelial cells, as nasal epithelium has been validated as a surrogate for distal lung epithelium in terms of ionic channel activity [12, 13]. We also explored differences related to the method of delivery. Our hypothesis stated that respiratory epithelia gene expression could be detected in GA obtained shortly after birth.

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2. Methods

2.1. Patients

This observational, proof-of-concept, cross-sectional study was conducted from November 2013 to April 2014, in three medical centers located in Northeastern Mexico: The Hospital Regional Materno Infantil, the Hospital Metropolitano Dr. Bernardo Sepúlveda and the Hospital Zambrano-Hellion. The first two centers are public hospitals with level II-III neonatal intensive care units that belong to the Servicios de Salud Network in Nuevo León, Mexico. The last center is a private hospital with a level III neonatal intensive care unit that belongs to the academic and research health branch of Tecnologico de Monterrey in Monterrey, Nuevo Leon, Mexico. The target population was composed of neonates born at term (gestational age $\geq$37 weeks) after an uneventful pregnancy. As this study had an exploratory, proof-of-concept design, a non-sequential and non-probabilistic convenience sample of about 30 inborn neonates of either sex delivered vaginally or by elective cesarean section was recruited. The inclusion criteria were: Inborn neonates; born at or above a gestational age of 37 weeks, either by vaginal delivery or elective cesarean section, without regard of gender. Exclusion criteria were: parental refusal of informed consent; evidence or history of use of corticosteroids, catecholamines or diuretics in the 7-day period before birth; neonates with a maternal history of preeclampsia or any other hypertensive disorder, urinary tract infection, chorioamnionitis, premature rupture of membranes, congenital anomalies, aneuploidy or any other maternal pathological condition that required hospitalization in the week before birth; and, for the babies born by elective cesarean section, onset of labor with cervical modifications prior to the delivery. Subjects who required respiratory support or supplementary oxygen during birth, and those for whom study samples could not be obtained, were also excluded. The study protocol was approved by the institutional human research review board at Escuela de Medicina, Tecnologico de Monterrey. Eligible neonates were included after written informed consent had been obtained from their parents or legal guardians. From the mother's charts we recorded maternal age, gestational age, presence of gestational diabetes, and blood group. For the newborns, we collected information on birth weight, sex, and method of delivery. We also registered adverse effects of sampling methods, adding nasal hemorrhage to the criteria outlined in Common Terminology Criteria for Adverse Events (CTCAE 4.03) catalog, NIH publication No. 09-5410, Revised and reprinted June 2010.

2.2. Sample collection

Samples were collected immediately after birth, by house staff physicians. The nasal samples were gathered under direct vision by scraping the nasal epithelium with an ASI Rhino-Probe® Curette (Arlington Scientific, Springville, UT, USA), as described previously [12]. GA was obtained by a gentle suction of the stomach

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contents through a sterile tube, collected in a sterile vial that contained 3 mL phosphate-buffered saline (PBS). Both samples were obtained from each study subject. Once gathered, samples were solubilized in PBS and then centrifuged for 5 min at 4,000 × *g* and 4 °C. Then supernatant was removed, and the cell pellet was dissolved in 800 μL of TRIzol® reagent and stored at −80 °C until RNA extraction.

2.3. RNA isolation

Total RNA was extracted from cell pellets according to the instructions provided by the TRIzol® reagent. Briefly, samples were resuspended in 1 mL of TRIzol® reagent and incubated at room temperature for 5 min. Chloroform (200 μL) was added and mixed with each suspension, and the sample was centrifuged for 15 min 12,000 × *g* and 4 °C. Total RNA was recovered from the supernatant with 1 mL isopropanol and then washed with 70% ethanol. RNA was solubilized in TE buffer. RNA concentration and purity were estimated by spectrophotometry, using a Nanodrop® 2000 device (Thermo Scientific, Wilmington, DE, USA) at a wavelength of 260/280 nm, and RNA integrity was assessed by electrophoresis on 1% agarose gels stained with ethidium bromide (Invitrogen, Life Technologies, Carlsbad, CA, USA). Isolated RNA was stored at −80 °C until analysis.

2.4. Sample quality assurance

Samples with ≥20 ng/μL total RNA and a 260/280 absorbance ratio ≥1.65 were included in the study. Quality was deemed to be good or poor when samples from both and neither site, respectively, fulfilled these criteria. Samples of poor quality were excluded from the final analysis. Samples that were grossly contaminated with meconium were discarded from the study.

2.5. Reverse transcription and quantitative PCR assays

From 150 ng total RNA, cDNA was prepared using the SuperScript III First-Strand Synthesis System for RT-qPCR® (Invitrogen Life Technologies). Briefly, total RNA was reverse transcribed in 21 μL of RT reaction buffer, containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 200 mM DTT, 10 mM of each deoxyribonucleotide triphosphate, 0.5 μg oligo dT, 200 U Superscript III reverse transcriptase, 40 U RNase OUT, and 2 U RNase H.

PCRs were carried out with the TaqMan® Universal PCR Master Mix (Perkin Elmer, Foster City, CA, USA). The primers and fluorogenic probes were designed using the Primer Express software from PerkinElmer Life Sciences. PCR were performed in a total volume of 25 μL containing 400 nM of each oligonucleotide, 200 mM of the TaqMan® probe, and 2 μL of cDNA at 1:16 dilution, in a 96-well reaction plate, on a 7500 Fast System® (Applied Biosystems, Foster City, CA,

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USA). Thermal cycling was initiated with an initial incubation at 50 °C for 2 min and 95 °C for 10 min. Then, 35 cycles, each consisting of heating at 95 °C for 15 s for denaturalization, followed by 1 min at 60 °C for annealing and extension, were performed. As an endogenous control, an r18s fluorogenic probe labeled with FAM® dye (Applied Biosystems) was analyzed in parallel. Each sample was run in triplicate. Negative controls were included in the same plate. Results were analyzed using the comparative threshold cycle (Ct) method, expressed as fold differences in gene expression relative to the endogenous control, and calculated as fold change $= 2^{-\Delta\Delta Ct}$ [14].

2.6. Statistical analysis

Values are expressed as means and standard deviations (SD); categorical data are expressed as frequencies and proportions. Student's t tests, the χ^2 test and Fisher's exact tests were used for comparison, as appropriate. P values <0.05 were considered to be statistically significant. The statistical analysis was done with IBM-SPSS® Statistics software (ver. 22; IBM Corporation, Armonk, NY, USA).

3. Results

Over the 6 months of the study period, a total of 1,449 live births occurred in the study hospitals. A convenience sample was recruited among those who fulfilled inclusion criteria, and whose parents agreed to participation by means of a written informed consent document; patient flow and eligibility are detailed in Fig. 1.

Baseline demographics and clinical characteristics of the study group were typical of a term newborn population. The sample was characterized by a strong

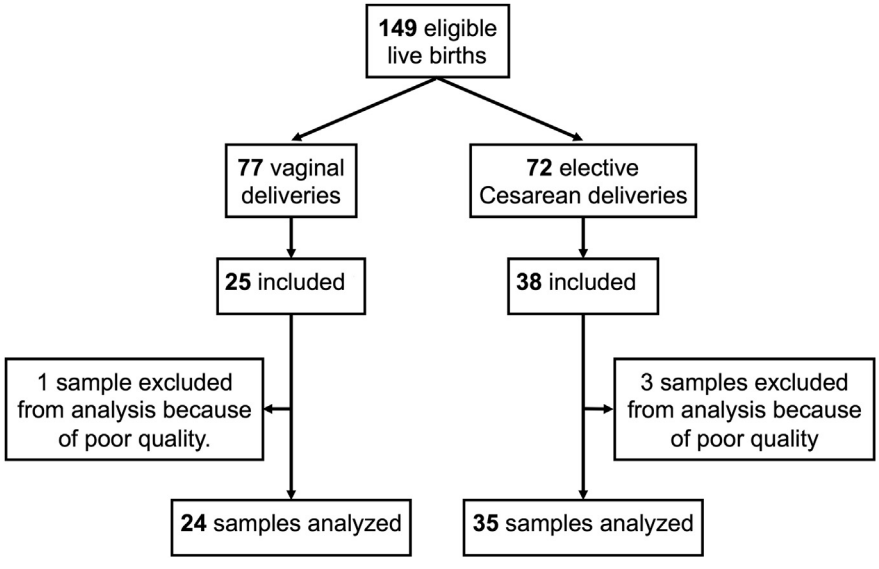

Fig. 1. Study flow chart.

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preponderance of females, as well as a greater proportion of babies born by cesarean section. None of the study subjects required respiratory support or more advanced resuscitation measures, and none developed a respiratory disorder after birth (Table 1).

The quality of samples was deemed as poor in four (6%) cases and good in 59 (94%) cases. Thus, after excluding poor-quality samples, final analysis was done in 94% of samples. Not all samples had amplification of both study genes by RT-qPCR assays.

The mean AQP5 expression detected in GA was significantly greater than that the expression in nasal mucous membrane samples. No difference was observed in the expression of the β-ENaC gene in GA and in nasal mucous membrane samples (Table 2).

When the study population was separated by method of delivery, significant differences in the relative expression of AQP5 were observed in both sample sites; expression was more than two-fold greater in GA of neonates delivered by cesarean section, and almost four-fold greater in nasal samples. For AQP5, relative expression was greater in GA than in nasal samples.

In contrast, the mean β-ENaC expression was two-fold greater in nasal samples of those born vaginally, while no difference was observed in GA samples. For β-ENaC, relative expression was similar between sample sites (Table 3).

Table 1. Demographics and clinical characteristics of term neonates included in the study n = 63.

	Value
Demographics	
Gender	
Male[a]	23 (37)
Female[a]	40 (63)
Method of delivery	
Vaginal[a]	25 (40)
Abdominal[a]	38 (60)
Gestational age, weeks.[b]	39 (1.1)
Maternal age, years.[b]	24.5 (6.1)
Clinical characteristics	
Birth weight, g[b]	3256 (430)
Birth length, cm[b]	50 (1.5)
Birth head circumference, cm[b]	34.5 (1.6)
Apgar score[c]	
1 minute	8 (7–9)
5 minutes	9 (9–10)

Annotations: [a] Values expressed as frequency and (percentage). [b] Values expressed as mean and (standard deviation). [c] Median (minimum – maximum).

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Table 2. Relative gene expression and yield in samples from nasal mucosa scrapping and gastric aspirate in term neonates, n = 59.

Gene of interest	Sample site				p value
	Nasal		Gastric		
	mean (SD)	Yield	mean (SD)	Yield	
AQP5	0.81 (1.0)	81	1.95 (2.3)	91	0.001[a] NS[b]
β-ENaC	0.9 (1.0)	81	0.7 (0.9)	90	NS[a] NS[b]

Annotations: AQP5 = Aquaporin 5; β-ENaC = Sodium epithelial channel 1, beta fraction. Values are expressed as $2^{-\Delta\Delta Ct}$ mean and (standard deviation) Yield = percentage of samples that yielded amplification. [a] Analysis done with Student's t test test, comparison was done on the values for gene expression; [b] Analysis done by Fisher's exact test, comparison was done on the values for sample yield; NS = Non-significant.

Although the rate of occurrence of adverse effects of the sampling methods was significant, as it affected 11 of 63 (17.5%) study subjects, the overall severity of these effects was low (grade 1 in the CTCAE v. 4.03, USDHHS, NIH, NCI) and no therapeutic intervention beyond observation was required. The most frequent adverse effect was discrete nasal bleeding in six (9.5%) subjects, followed by discrete gastric bleeding in four (6.3%) subjects and both discrete nasal and gastric bleeding in one (1.6%) of the 63 included subjects. The presence of blood staining in samples did not interfere with the performance of the test.

Table 3. Relative gene expression and yield in samples from nasal mucosa scrapping and gastric aspirate, separated by method of delivery, in term neonates, n = 59.

Gene of interest/Sample site	Method of delivery				p value
	Vaginal		Abdominal		
	mean (SD)	Yield	mean (SD)	Yield	
AQP5					
Nasal	0.3 (0.3)	88	1.2 (1.1)	77	0.001[a] NS[b]
Gastric	1.1 (1.4)	96	2.5 (2.7)	88	0.02[a] NS[b]
β-ENaC					
Nasal	1.3 (1.1)	88	0.6 (0.6)	77	0.03[a] NS[b]
Gastric	0.5 (0.5)	96	0.8 (1.0)	86	NS[a] NS[b]

Annotations: AQP5 = Aquaporin 5; β-ENaC = Sodium epithelial channel 1, beta fraction. Values are expressed as $2^{-\Delta\Delta Ct}$ mean and (standard deviation) Yield = percentage of samples that yielded amplification; [a] Analysis done with Student's t test, comparison was done on the values for gene expression; [b] Analysis done by Fisher's exact test, comparison was done on the values for sample yield; NS = Non-significant.

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4. Discussion

Although the need for routine gastric aspiration of normal-term babies in the delivery room born from uncomplicated pregnancies has been strongly questioned, and some adverse effects of this procedure on physiological parameters have been identified [15], it differs for ill neonates. In this context, the utility of GA has been established, along with the history of polyhydramnios and other clinical signs, as an aid in the early detection of occlusive anomalies of the digestive tract [16]. GA has been proven to contribute to the microbiological diagnosis of congenital tuberculosis and neonatal sepsis [17] and, more recently, due to its sensitivity, ease of use and relatively low cost, as a feasible and practical method for the identification of pathogens to which neonates may have been exposed to in utero [18, 19]. Moreover, in the early 70′s of last century, Yeung and Tam demonstrated that GA obtained from non-infected neonates contains a mix of different cells, of which around 62 or 90% could be identified, based on their morphology, as of epithelial origin, be it either gastric or respiratory [20].

A few years after, other research groups established the utility of GA in the diagnosis of RDS, focusing on biophysical and biochemical parameters; GA analysis has been identified as a strong diagnostic and predictive tool for the neonatal RDS [21, 22, 23], with a diagnostic efficacy comparable to that of tracheal aspirate. It has been shown also that GA obtained in the immediate postpartum period, within 30 minutes after birth contains swallowed amniotic fluid as well as fetal lung fluid, with phospholipids derived from the respiratory tract, moreover, when amniotic fluid samples are not available, GA is a convenient source in which to estimate respiratory maturity indexes [22, 23, 24]. In 2007 Kaeffer *et al.* explored gene expression of gastrointestinal tract epithelia from gastric residual fluid or stool samples collected from preterm infants to investigate the impact of therapeutic and nutritional regimens on the maturation of gastrointestinal functions. They found a significant number of exfoliated epithelial cells in GA [25]. In 2011, the same group established GA as a relatively noninvasive source of neonatal gastric epithelium, concluding that cells harboring markers of a progenitor status can be recovered from GA in preterm infants to measure the expression of specific genes of interest [26]. These works prompted us to explore the possibility of identifying genes specific to respiratory epithelium, assumed to be conserved in the gastric contents obtained within 30 min after birth.

Our selection of AQP5 and β-ENaC genes was based on the expression of AQP5 in the apical membrane of type I alveolar epithelial cells, in the apical membrane of acinar epithelial cell in submucosal glands and also in a subset of airway epithelial cells, while being absent in normal gastric or esophageal tissues [27]; and the proven expression of β-ENaC, along with the subunits α and γ, in distal respiratory epithelia [7, 8, 10]. Although earlier studies in an AQP5 knockout mice model challenged the extent of the role of AQP5 in removing an excess of acutely instilled fluid [28], based

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on the possibility that chemical knock-out could trigger possible compensatory changes in organ function, currently it is accepted that both aquaporins AQP1 and AQP5 provide the principal route for osmotically driven water transport between airspace and capillary compartments, and AQP5 deletion in submucosal glands reduces fluid secretion by >50% [8, 9, 29, 30, 31, 32, 33]. Also, the roles of AQP5 and β-ENaC abnormalities in the genesis of human neonatal respiratory disorders have been already clearly established [5, 6, 7, 8, 9, 10]. Helve et al clearly demonstrated, using nasal epithelial samples, that preterm infants with RDS have markedly lower levels of α-, β-, and γ-ENaC mRNA relative to healthy term infants, and the positive effect that glucocorticoids exert on that expression, thus establishing the role of ENaC low expression to the development of RDS [10, 30, 31, 32, 33].

Our findings prove for the first time the feasibility of identifying and quantifying the expression of AQP5 and β-ENaC in GA, obtained shortly after birth from term newborns. In addition, the rate of gene expression observed in GA samples was comparable to that found in scraped nasal epithelial cells, which have been validated previously as a surrogate for distal respiratory epithelia in terms of ionic channel activity and gene expression [12, 13]. These findings are the main contributions of our work. GA collection is easier than nasal potential difference performance or nasal scrapping [12, 13], and although it may be considered an invasive procedure, is certainly a much less invasive approach than a tracheal aspiration or bronchoalveolar lavage [7], which could be justified only for the most severely ill neonates, who require tracheal intubation. Moreover, GA could become a suitable source to estimate respiratory maturity in the absence of amniotic fluid samples, due to amniotic fluid loss (like in severe oligohydramnios or premature rupture of the membranes) or inability to collect a convenient sample (gross meconium or blood amniotic fluid contamination).

A thorough description of fetal lung fluid electrolyte composition and how it changes along modifications in AQP5 and ENaC is beyond the scope of our work, those interested should consult the excellent review published by Bland and Nielson in 1992 [34].

Our data also confirm previous observations that β-ENaC expression is greater in nasal scrapings of those babies delivered vaginally, as described first in small and medium mammals and later confirmed in human neonates [30, 35].

Previous researchers have found that α-ENaC expression could be detected as early as the embryonic period [30], although others reported that expression of the β and γ subunits of these genes in the conductive and respiratory airway epithelium, serous cells, Clara cells, and alveolar type II cells is restricted to the canalicular stage of lung development (17−24 weeks), increases late in gestation, keeps a close relationship to length of gestation and, after birth, also with postnatal age [10, 31, 32, 33].

Recently, a sex difference in alveolar epithelial sodium transport has been described in fetal distal lung epithelia in rats [36]. Although no human study has addressed this

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issue, a potential limitation of our study is that our population consisted of nearly twice the number of female infants as compared to male infants; however, adjustment for this factor did not modify the results of the analysis.

As our population was composed only of term neonates, notwithstanding the differences observed regarding AQP5 and β-ENaC, either by mode of delivery and by sample site, these differences were not accompanied by abnormalities in respiratory function, perhaps these values should be seen better as a reference pattern of the normal expression of these genes. We are aware that our approach is limited by the inability to generalize and translate our findings in term newborns to preterm neonates; to address this issue, we are currently exploring this topic in a larger cohort of early and late-preterm neonates. If confirmed, this approach would constitute an additional method for the estimation of respiratory epithelial gene expression in that population, to evaluate the effectiveness of lung fluid clearance, and perhaps be used to predict the two most common forms of non-infective neonatal respiratory disorders.

In conclusion, we demonstrated that the expression of AQP-5 and β-ENaC genes can be evaluated in GA material recovered from term neonates, with levels comparable to those found in scraped nasal epithelium. As in many cases a gastric tube will likely be necessary for clinical reasons in a sick neonate, GA analysis allows the practitioner to avoid more invasive approaches, such as bronchoalveolar lavage, in the search for the etiology of the two most common non-infective neonatal respiratory disorders.

Declarations

Author contribution statement

F. Castorena-Torres: Conceived and designed the experiments; Performed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

M. Alcorta-García: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

V. Lara-Diaz: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

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Additional information

The study described in this paper was registered at Secretaría de Salud. Gobierno del Estado de Nuevo León, México. Directiva No. DEISC-INVEST-189/2016, Dirección de Enseñanza, Investigación en Salud y Calidad under the registration number DEISC-19 01 16 22.

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