Journal of Perinatology (2013) 33, 15–20

www.nature.com/jp

ORIGINAL ARTICLE

Usefulness of serum lipid peroxide as a diagnostic test for hypoxic ischemic encephalopathy in the full-term neonate

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Objective: To evaluate the usefulness of serum lipid peroxide (LPO) for hypoxic ischemic encephalopathy (HIE) in full-term neonates.

Study Design: Diagnostic test evaluation forming three groups: (1) healthy full-term neonates ($n = 59$), (2) at-risk full-term neonates without HIE ($n = 57$) and (3) at-risk full-term neonates with HIE ($n = 57$). HIE diagnosis was made using the Finer clinical classification at 48 h after birth. Serum LPO was taken at 4 h after birth and determined by spectrophotometry.

Result: One hundred seventy-three full-term neonates were studied. Fifty-one of the at-risk full-term neonates with HIE (51/57) had high serum LPO and two of the at-risk full-term neonates without HIE (2/57) ($P<0.001$). Serum LPO level had 89% sensitivity, 96% specificity, 96% positive predictive value, 90% negative predictive value, 24 positive probability ratio, 0.11 negative probability ratio and 92% diagnostic usefulness.

Conclusion: Serum LPO level could be a useful test for early diagnosis of HIE in full-term neonates.
Journal of Perinatology (2013) **33,** 15–20; doi:10.1038/jp.2012.38; published online 5 April 2012

Keywords: neonate; Apgar; Finer; sensitivity; specificity; Mexico

Introduction

Perinatal asphyxia affects the central nervous system manifesting itself with clinical data of hypoxic ischemic encephalopathy (HIE) that can have incapacitating neurological consequences.[1] Incidence is 3 per 1000 live births,[2] mortality is from 15 to 20%, and 25% of survivors suffer permanent neurological deficiency.[1]

Perinatal asphyxia is caused by factors that inhibit the transfer of oxygen from the mother to the fetus and that reduce the passing of carbon dioxide (CO_2) from the fetus to the mother.[3] Oxygen deprivation at birth can cause two types of events: hypoxia and/or ischemia, the latter of which is important in brain injury origin and is considered to be the most important physiopathological factor for developing HIE.[4] After ischemia, there is reperfusion from the administration of supplementary oxygen. All these events in the brain of the full-term neonate provoke an imbalance between increased free radical production and antioxidant deficiency (oxidative stress), causing neuronal injury.[5,6]

Certain methods for diagnosing fetal hypoxia are described in the literature. These methods are based on findings present at birth: (1) metabolic or mixed acidosis with pH <7.0; (2) Apgar score under 3 at 5 min after birth; (3) neurological symptoms in the neonatal period (convulsions, coma and hypotonia) and (4) multiple system dysfunction.[7,8] HIE diagnosis based on the Finer classification[9] cannot diagnose disease until 48 h after the hypoxic/ischemic events have occurred.

Another method for early identification of HIE secondary to perinatal asphyxia causing hypoxic/ischemic events by generating oxidative stress (free radical increase with antioxidant deficiency) is the measuring of degradation of free radicals that react with unsaturated fatty acids in the lipid membrane, causing lipid peroxidation that produces membrane damage and necrosis in neuronal cells.[6] Ogihara *et al.*[10] observed a lipid peroxidation increase in cerebrospinal fluid at 72 h after perinatal asphyxia event. Schmidt *et al.*[11] observed lipid peroxide (LPO) elevation in full-term neonates with perinatal asphyxia in blood samples from the umbilical artery at birth.

It has been demonstrated that some anesthetic agents, including halotane, reduce oxidative stress manifestation.[12] Propofol does

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Received 1 August 2011; revised 6 January 2012; accepted 21 February 2012; published online 5 April 2012

not increase reactive substances after its administration and has antioxidant properties.[13,14] Phenobarbital reduces lipid peroxidation levels in neonates with perinatal asphyxia,[15] and alcohol and smoking reduce serum perioxidation level.[16] Bilirubin is a potent antioxidant that diminishes oxidative stress by inhibiting lipid peroxidation.[17]

To the best of authors' knowledge, there are no studies in the literature associating serum LPO level with clinical stages of HIE secondary to perinatal asphyxia and therefore the objective of this study was to evaluate the usefulness of LPO level determination as a diagnostic test for HIE in the full-term neonate.

Methods

The study was carried out from February 2007 to January 2009 in two metropolitan specialized hospitals with neonatal intensive care units in Guadalajara, Mexico (*Hospital de Ginecología y Obstetricia* of the *Instituto Mexicano del Seguro Social* and the *Hospital Materno Infantil Esperanza López Mateos* of the *Secretaría de Salud*).

Study design

A comparative cohort study for diagnostic test evaluation was designed.

Inclusion and exclusion criteria

Healthy full-term neonates and neonates at risk for HIE according to the following factors were included in the study: HIE risk was evaluated by Apgar score obtained 5 min after birth and by the need to carry out resuscitation maneuvers at birth.

HIE was evaluated with the modified Sarnat-Sarnat clinical classification and was classified into three stages according to clinical manifestations present at 48 h after birth.[18] Full-term neonates that fit the following criteria were excluded from the study: (1) major congenital malformations; (2) indirect bilirubin levels $>15\,mg\,dl^{-1}$; (3) congenital metabolic disease data; (4) neonates whose mothers had history of addiction; and (5) neonates whose mothers had received general anesthesia during birth process.

Sample size

Sample size was calculated using the formula for estimating proportion based on 95% specificity, according to a publication by Schmidt *et al*.[11] It was defined as the probability of serum LPO being increased when the neonate really had HIE. A 95% confidence interval and 6% precision were used, resulting in a total of 51 neonates per group. This was increased 10% to compensate for probable losses, resulting in a sample of 56 neonates per group. Consecutive sampling was employed.

Study development

One hundred seventy-three neonates were evaluated by a neonatologist at birth. Information was registered in a structured questionnaire and the following data were evaluated:

(1) *Neonate clinical characteristics.* The following antecedents were studied: (1) maternal addictions and type of anesthesia employed during birth process and (2) type of birth, Apgar score at first minute and at 5 min after birth, whether or not cardiopulmonary resuscitation was needed. This was defined when any of the following maneuvers were used: supplementary oxygen with face mask and self-inflating bag with positive pressure, cardiac massage and medications. In addition, vital signs, Capurro clinical evaluation,[19] and weight and height were determined and 1 ml of blood was taken for neonatal metabolic screening.

(2) *Risk for HIE development.* Risk was considered to exist with Apgar score $\leqslant 6$ at 5 min after birth or when there was the need for cardiopulmonary resuscitation according to the neonatal cardiopulmonary resuscitation manual.

(3) *Finer clinical classification for HIE.*[9] This classification was analyzed by a neonatologist in all full-term neonates 48 h after birth to evaluate the presence of HIE. HIE was then classified into three stages according to clinical manifestations present: (1) (slight): hypervigilance, hyperreflexia, dilated pupils, tachycardia and absence of convulsions; (2) (moderate): lethargy, hyperreflexia, miosis, bradycardia, convulsions; hypotonia with weak sucking and Moro reflex; (3) (serious): stupor, flaccidity, reduced pupils and scant reaction to light in middle position, reduced myotatic reflexes, hypothermia and absence of Moro reflex. In relation to this evaluation, Apgar score at birth, and cardiopulmonary resuscitation antecedent, three full-term neonate groups were formed: (1) healthy neonates (2) neonates at risk for HIE but without HIE and (3) neonates at risk for HIE and presenting with HIE.

(4) *LPO determination.* A 1-ml peripheral venous blood sample was taken from all neonates included in the study at 4 h after birth. It was centrifuged at 2500 r.p.m. for 5 min and frozen at $-70\,^{\circ}C$ for a maximum of 6 months. All determinations were carried out simultaneously according to manufacturers' instructions.[3] K-Assay-LPO-CC from the Kamiya Biomedical Company (Seattle, WA, USA) was used for quantitative LPO determination. The process was carried out in a darkroom at room temperature. Spectrophotometry results were obtained at 675 nm by using an Omheda Medical (Laurel, MD, USA) model BJF651 apparatus. Determinations were carried out by a laboratory technician who was blinded to full-term neonate clinical diagnosis.

According to laboratory results, normal LPO values were $<3.35\,nmol\,ml^{-1}$ and were the values found in the 95th percentile of the data distribution curve in the healthy full-term neonate group.

Table 1 Clinical characteristic comparison of full-term neonates in groups with and without HIE

Characteristic	*Healthy group* n = *59*	*At-risk group without HIE* n = *57*	*P1 value*	*At-risk group with HIE* n = *57*	*P2 value*
Weight in kilograms, median (range)	3.3 (2.5–4.4)	3.3 (2.1–4.6)	0.229	3.2 (2.0–4.8)	0.430
Height in centimeters, median (range)	50 (48–56)	51 (45–56)	0.699	50 (46–55)	0.197
Sex, male, *n* (%)	31 (52)	39 (68)	0.810	39 (70)	0.580
Capurro in weeks, median (range)	39 (37–42)	40 (37–42)	0.100	39 (37–42)	0.125
Apgar score $\leqslant 6$ at 5 min, *n* (%)	0 (0)	1 (2)	0.490	30 (53)	<0.001
Birth by cesarean section, *n* (%)	9 (15)	27 (47)	<0.001	41 (73)	<0.05
Meconium in amniotic fluid, *n* (%)	4 (7)	23 (40)	<0.001	23 (41)	0.682
Hospital stay, median (range)	1 (1–4)	2 (1–9)	0.125	5 (1–14)	<0.001
Mortality, *n* (%)	0 (0)	0 (0)	0.116	15 (27)	<0.001
Maternal age in years, median (range)	22 (15–44)	22 (14–40)	0.765	26 (15–40)	<0.05

Abbreviations: HIE, hypoxic ischemic encephalopathy; *P*1, comparison of healthy group with at-risk group without HIE; *P*2, comparison of the two at-risk groups, one with and one without HIE; Risk, Apgar $\leqslant 6$ at 5 min or cardiopulmonary resuscitation.
Mann–Whitney *U*-test was used for median comparison and χ^2-test was used for proportion comparison. Capurro: clinical evaluation for gestational age.

Statistical analysis

Usefulness value of high LPO levels for HIE diagnosis was calculated using the Finer classification as criterion standard. Sensitivity, specificity, positive predictive value, negative predictive value, positive probability ratio and negative probability ratio, along with 95% confidence intervals, were calculated. Sensitivity was defined as the proportion of full-term neonates with HIE with elevated LPO, specificity as the proportion of full-term neonates without HIE that did not have elevated LPO, positive predictive value as the proportion of full-term neonates with elevated LPO and HIE, and negative predictive value as the proportion of full-term neonates with normal LPO and without HIE. Positive probability ratio was defined as the most probable number of times elevated LPO level would present in a full-term neonate with HIE rather than in a full-term neonate without HIE and negative probability ratio as the most probable number of times normal LPO level would present in a full-term neonate without HIE rather than in a full-term neonate with HIE. Exactitude was obtained by adding the correct and incorrect diagnostics and dividing the result by the total number of neonates. Interobserver variability of laboratory LPO determination was calculated by the Kappa index of agreement producing a value of 0.92. LPO results presented an abnormal distribution curve and so non-parametric statistics were used. Mann–Whitney *U*-test was used to compare quantitative variables between two groups and χ^2-test was used to compare proportions. Statistical significance was considered to exist when $P<0.05$. SPSS version 13.0 (Chicago, IL, USA) statistical package was used.

Ethical considerations

This study followed the ethical principals for scientific research contained in the World Medical Association Declaration of Helsinki.[20] Informed consent forms were signed by the parents of the neonates. The study was accepted at both hospitals by their research and ethics committees with the following numbers: *Instituto Mexicano del Seguro Social* 2004/1310/0024 and *Secretaría de Salud* 166743/239.

Results

One hundred seventy-three full-term neonates were analyzed and formed three groups: (1) healthy full-term neonates ($n = 59$); (2) full-term neonates at risk for HIE ($n = 57$) but not presenting with HIE; and (3) full-term neonates at risk for and presenting with HIE ($n = 57$). Of the at-risk neonates with HIE, 51 (89%, 51/57) had elevated LPO. Of the at-risk neonates without HIE, 2 (3%, 2/57) had elevated LPO. In the neonate group with HIE, the HIE was classified into stage I ($n = 23$) 40%, stage II ($n = 18$) 32% and stage III ($n = 16$) 28%.

Table 1 shows the clinical characteristics of the full-term neonates included in the study. Statistically significant differences were observed when comparing the normal group with the at-risk group without HIE and with the at-risk group with HIE. In the respective comparison of the at-risk group without HIE and the at-risk group with HIE, the HIE group had older maternal age (22 vs 26) ($P<0.050$), a high proportion of Apgar score $\leqslant 6$ at 5 min after birth (1 vs 30) ($P<0.001$), a high proportion of births by cesarean section (27 vs 41) ($P<0.001$) and a high proportion of mortality (0 vs 15) ($P<0.05$).

Figure 1 shows LPO concentration comparison among the different groups. There was elevated LPO in the at-risk with HIE group compared with the healthy neonate and at-risk without HIE neonate groups. Median value and range were expressed in nmol ml^{-1} as follows: normal healthy group, 1.1 (0.3 to 2.8); group without HIE, 1.2 (0.5 to 4.0); and group with HIE, 9 (0.7 to 12.5).

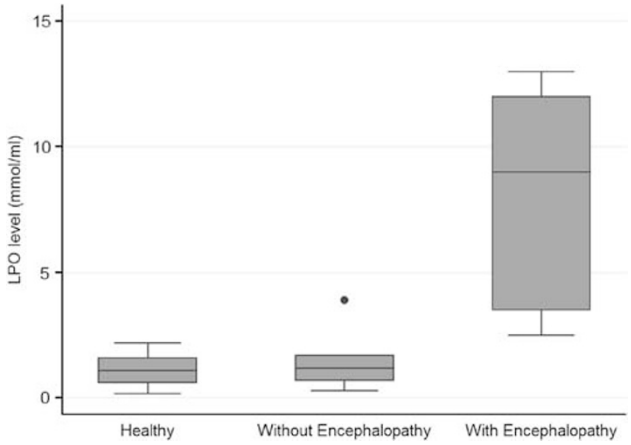

Figure 1 Lipid peroxide (LPO) level comparison of healthy group with the two at-risk groups with and without hypoxic ischemic encephalopathy (HIE) in full-term neonates. Mann–Whitney *U*-test was used for median comparison. Comparison of healthy group with at-risk group without HIE $P = 0.36$, comparison of at-risk groups with and without HIE $P = 0.001$, comparison of healthy group with at-risk group with HIE $P = 0.001$. •Max outlier.

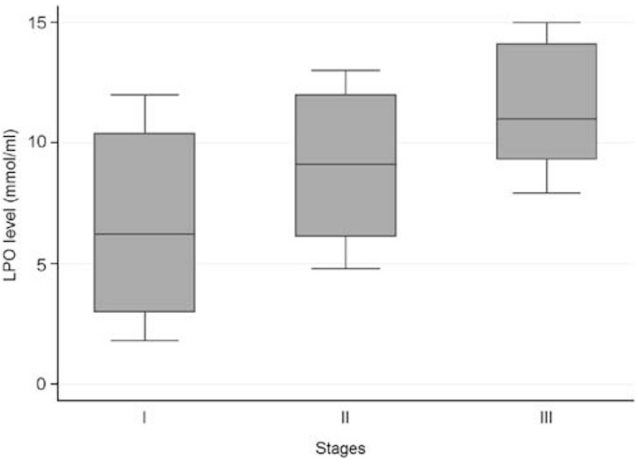

Figure 2 Lipid peroxide (LPO) level comparison of the three clinical stages of the Finer clinical classification for hypoxic ischemic encephalopathy (HIE) in full-term neonates. Mann–Whitney *U*-test was used for median comparison. Stage I and stage II comparison $P = 0.98$, stage II and stage III comparison $P = 0.03$, stage I and stage III comparison $P = 0.001$.

Figure 2 shows LPO level distribution in the HIE group when subdivided into the three Finer clinical classification stages. There was higher concentration of LPO level in stage III when compared with stage II ($P = 0.03$) and stage I ($P = 0.001$), and these results showed statistically significant difference.

Table 2 shows the usefulness value of LPO level using the Finer clinical evaluation results for HIE diagnosis as the golden standard. LPO level had high sensitivity (89%), specificity (96%), positive predictive value (96%) and negative predictive value (90%).

Table 2 LPO usefulness for supporting HIE diagnosis in full-term neonates

Characteristic	*Value*	*95% CI*
Prevalence	50	41–59
Exactitude	92	86–96
Sensitivity	89	82–97
Specificity	96	92–100
Positive predictive value	96	91–100
Negative predictive value	90	83–98
Positive probability ratio	24	6.1–94.4
Negative probability ratio	0.11	0.05–0.23

Abbreviations: 95% CI, 95% confidence interval; HIE, hypoxic ischemic encephalopathy; LPO, lipid peroxide.
Lipid peroxide cutoff point: 3.35 nmol/ml, true positives 51, true negatives 55, false positives 2 and false negatives 6.

Discussion

High LPO sensitivity, specificity, positive predictive value and negative predictive value were observed in the present study for identifying HIE in full-term neonates with low Apgar score or the need for cardiopulmonary resuscitation at birth. These data are important for supporting early diagnosis and being able to opportunely initiate therapeutic measures for preventing mortality and long-term neurological consequences.

To the best of authors' knowledge, there are no studies in the literature evaluating LPO level in different HIE clinical stages caused by perinatal asphyxia.

HIE diagnosis is difficult to identify through the presence of clinical neurological manifestations in the first 24 h after perinatal asphyxia event. The Finer classification is useful in evaluating clinical neurological alterations after obtaining a low Apgar score. However, an important limitation of this classification is the >48-h waiting period after perinatal asphyxia event that is needed to obtain results that support HIE diagnosis.[9]

LPO determination is a biochemical test for HIE diagnosis that provides results within the first 72 h after an asphyxia event at birth.[6]

Ogihara *et al*.[10] measured $F_{2(alpha)}$-isoprostane as a lipid peroxidation marker during the first 72 h after birth in the cerebrospinal fluid of 10 full-term neonates with HIE and compared the results with full-term neonates with no perinatal asphyxia antecedents in whom lumbar puncture had been performed to exclude meningitis as part of sepsis or infection suspicion. This marker was found to be more elevated in full-term neonates with HIE than in the control group (117.3 ± 67.1 vs 65.9 ± 28.0, $P = 0.001$). However, no significant correlation was found with HIE clinical stages ($r = 0.58$, $P = 0.1$). There were similar results in the present study. Elevated LPO level was found in the first 4 h after birth in the full-term neonates with low Apgar score or need for cardiopulmonary resuscitation. In addition, there

was significant correlation when LPO level was found to be elevated in stage III of the Finer clinical classification for HIE diagnosis when compared with stage I and stage II. This discrepancy with the Ogihara study was probably due to the fact that the blood sample for determining LPO was taken in the first 4 h after asphyxia event in the present study and in the Ogihara study the sample was taken from cerebrospinal fluid up to 72 h after asphyxia event. In addition, sample size was larger in the present study.[21,22]

Schmidt *et al*.[11] studied five groups of neonates, of which three were full-term neonates, and put them into three groups: (1) healthy; (2) with acidosis (umbilical artery pH <7.20) and without asphyxia, defined by Apgar score above 8 points 5 min after birth; and (3) with acidosis and asphyxia defined by Apgar score under 7 at 5 min after birth. LPO product level in blood plasma from the umbilical cord artery at birth was measured in those three groups. LPO elevation was twice as high in the full-term neonate group with acidosis and asphyxia when compared with the healthy full-term neonate control group and those results were statistically significant. However, there was no correlation with HIE clinical data.[11] These results are similar to those of the present study in which LPO elevation was higher in the full-term neonates with Apgar score $\leqslant 6$ or need for cardiopulmonary resuscitation. In addition, the present study compared LPO results with the different clinical stages of the Finer clinical classification for HIE diagnosis and showed that when HIE stage was higher, LPO level was also higher.

In the present study, serum LPO sample was taken 4 h after birth because some authors, in their experimental designs, have established 6 h as the optimum time for therapy such as hypothermia. For this reason, serum LPO level was studied before initiating any HIE treatment.[23,24]

One of the limitations in the present study was that serum LPO was evaluated as a diagnostic test for HIE; and therefore, no response to treatment or long-term neurological development was analyzed.

The results of the present study lend support to the usefulness of LPO level for diagnosing HIE and several of its advantages are ease for taking venous blood sample, a small quantity of blood is required, it is easy to determine, it is low cost, and most importantly, LPO result can be obtained in the first 4 h from the time the full-term neonate presents with asphyxia event. These advantages lend support to the view that LPO is a biochemical marker that can indicate the existence of neuronal alteration early on and therefore with a degree of alteration different from what it would be after waiting for clinical manifestation, using Finer scale classification, and subsequent HIE diagnosis 48 to 72 h after the asphyxia event. However, LPO has the disadvantage of requiring a specialized technique carried out by trained laboratory personnel.

It can therefore be concluded that determining LPO level could be a useful test for early HIE diagnosis in the full-term neonate. LPO level elevation is directly proportional to HIE stage elevation. It would also be of interest to carry out studies on the temporal behavior of the LPO curve in HIE.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

We wish to thank the Centro Universitario de Investigaciones Biomédicas of the Universidad de Colima and the Departamento de Inmunología of the Centro de Investigaciones Biomédicas de Occidente in Guadalajara, Mexico, and in particular, Georgina Hernández PhD.

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