Changes in S100B and troponin levels in a fetal sheep model of worsening acidosis

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Yasmine Hamoud[1], Jean David Pekar[2], Elodie Drumez[3], Laure Lacan[4], Patrice Maboudou[2], Julien De Jonckheere[5], Laurent Storme[6], Véronique Houfflin-Debarge[1], Dyuti Sharma[7], Charles Garabedian[1], Louise Ghesquière[1]

ABBREVIATIONS

HIE: hypoxic-ischemic encephalopathy

HI: hypoxia-ischemia

FHR: fetal heart rate

c-TnT: cardiac troponin T

UCO : umbilical cord occlusion

ABSTRACT

Background - S100B and cardiac troponin T (c-TnT) are relevant biomarkers at birth of hypoxic-ischemic encephalopathy (HIE) and myocardial ischemia secondary to metabolic acidosis during labor, respectively. The purpose was to assess in-utero changes in S100B and c-TnT levels in an experimental model of labor-like acidosis.

Methods - Repeated umbilical cord occlusions (UCOs) in ten experiments were performed in mild (phase A, 1 UCO/5 mn), moderate (phase B, 1 UCO/3 mn), and severe (phase C, 1 UCO/2 mn) period. The experiments were stopped if arterial pH reached 6.90.

Results - UCOs resulted in fetal acidosis with pH dropping to 6.99 $\pm$ 0.13. When compared to the baseline period fetal S100B increased between phases A and B (7% ± 4 vs 17% ± 13, p = 0.030) and between phases A and C (7% ± 4 vs 24% ± 8, $p < 0.001$). Fetal c-TnT serum levels increased during occlusions: 102 ng/L (58-119) in phase A, vs 119 ng/L (103-198) in phase B vs 169 ng / L (128-268) in phase C ($p<0.05$, for all). When compared to the baseline control period, fetal ΔcTnT was significantly modified throughout UCO series: 5.0% (-3; 45) in phase A, 51% (4; 263) in phase B, and 77% (56.5; 269) in phase C ($p< 0.05$ for all).

Conclusions - S100B and c-TnT increased when fetal acidosis occurred, which reflects the potential neurological damage and fetal cardiovascular adaptation.

Key words: S100B, cardiac troponin T, acidosis, fetal sheep, biomarker, perinatal asphyxia

Introduction

Uterine contractions can reduce uteroplacental perfusion by up to 60% leading to progressive fetal hypoxia (1). At term, healthy fetuses are able to withstand this transient hypoxia by reducing oxygen consumption and redirecting the oxygenated blood flow to critical organs (heart, brain, and adrenals) through activation of the peripheral chemoreflex (2,3). Providing there is adequate time for fetoplacental reperfusion between contractions, this mechanism protects fetuses from severe and irreversible hypoxic injuries. However, some fetuses develop intrapartum metabolic acidemia which remains a leading cause for neonatal complications, such as stillbirth, hypoxic-ischemic encephalopathy (HIE) and cerebral palsy.

In the past decade, tissue-specific biochemical damage markers, such as S100B and cardiac troponin T (c-TnT) have been examined in umbilical cord blood at birth (4,5). The calcium-binding S100B protein is mainly concentrated in the glial cells of the nervous system. S100B has been presented as the most promising neurobiomarker in neonates with birth asphyxia (6). The increase in S100B concentration in body fluids, such as cerebrospinal fluid, blood, or urine, is a reliable marker of brain damage following asphyxia in neonates (7). The advanced hypothesis is that in case of brain lesions, some of the S100B released by the affected tissue could spread through the blood-brain barrier into the systemic circulation. In neonates suffering from severe forms of HIE, S100B is elevated in umbilical cord blood samples at birth and is associated with the severity of disease and risk of further neurodevelopmental sequelae (8). Cardiac troponin T is commonly used as a myocardial ischemia marker in both adults and infants. Studies have shown that in perinatal asphyxia, it could also be a strong and useful predictor of myocardial injury in birth asphyxia and HIE (9). Costa et al. reported that asphyxiated neonates had higher c-TnT levels associated with decreased cardiac output when compared with control healthy newborns (10). Yet, the degree of prematurity, fetal weight and type of delivery may complicate the assessment (11).

57 The in-utero levels of these biomarkers in response to worsening acidosis have still not been
58 described. The purpose of this study was to assess the changes in S100B and c-TnT levels in
59 both fetal and maternal serums in an experimental model of severe labor-like acidosis.

60 Materials and methods

61 *Surgical preparation*

62 Near-term pregnant sheep (Ile de France, INRA, Val de Loire, France) with a gestational age
63 of 124 ± 1 days (term = 145 days) underwent our surgical procedure, as described previously
64 (12,13). If an ewe had multiple fetuses, only one was instrumented and included in the
65 experiment.

66 For anesthesia, ewes had a vascular infusion of 500 mL of Ringer lactate, then a
67 premedication of 0.3 mL intravenous Sedaxylan (xylazine 20mg/ml; Dechra, The
68 Netherlands). Induction was made with isoflurane 5% before intubation, and anesthesia was
69 maintained with soflurane 2% (14). After maternal laparotomy and hysterotomy, catheters
70 (4-Fr diameter; Arrow, Reading, PA, USA) were placed in the fetal axillary arteries. The
71 catheter tips were advanced to the ascending aorta. Four electrocardiogram (ECG) electrodes
72 (Mywire 10; Maquet, Rastatt, Germany) were placed on the fetal intercostal muscles to
73 record fetal ECG. An inflatable silicone occluder (OC16; In Vivo Metric, Healdsburg, CA,
74 USA) was placed around the umbilical cord and the volume of saline solution required to
75 achieve complete occlusion was determined.

76 The ewes were acclimatized to the experiment room 1 hour a day in the presence of the main
77 investigators in charge of carrying out the experiments.

78 The experimental protocol began 4 days after the surgery. After 1 hour of baseline period,
79 repetitive umbilical cord occlusions (UCOs) were performed by injecting an isotonic solution
80 into the occluder to obtain a total occlusion for 1 minute. The protocol was divided into three
81 periods of 1 hour each, as described previously by Prout et al (15). During the mild phase,
82 UCOs were repeated every 5 minutes (phase A). During the second phase (phase B), the
83 rhythm was of one UCO every 3 minutes. Finally, the last period (phase C) consisted of one

UCO every 2 minutes. The protocol was stopped before the end of phase C if pH reached 6.90 or below.

Euthanasia was performed either at the end of the experimental procedure, or in cases of spontaneous labor, in-utero fetal death, or death during surgery, by intravenous injection of 6 mL/50 kg of T61 (1 mL contains embutramide 200mg + mebezonium 26.92 mg + tetracaine 4.39 mg, MSD France, Courbevoie, France) (14).

Monitoring parameters and measurement techniques

Arterial blood gas parameters were measured with the i-STAT 1 blood analyzer (i-STAT 1 System; Abbott Point of Care, Inc, Princeton, NJ, USA) using CG4+ cartridges at specific time points. Hemodynamic data were recorded continuously throughout the experiment. The fetal arterial and intra-amniotic catheters were connected to pressure sensors (Pressure Monitoring Kit 1; Baxter, Guyancourt, France) that were connected to a blood pressure monitor (Monitor Merlin, Hewlett Packard, Palo Alto, CA, USA). The arterial blood pressure (ABP) was measured from the blood pressure phasic signal and referenced to the intra-amniotic pressure (IAP) (calculated ABP = observed ABP - observed IAP). Biochemical assays were performed from serum on a Cobas 8000 module e601 (Roche, Basel, Switzerland) using the electrochimiluminescence method. S100B and c-TnT were measured in both the mother (M S100B or M c-TnT) and fetus (F S100B or F c-TnT). Collected blood samples were centrifuged at 3500 g for 10 minutes and supernatant stored at - 80° C until assessment. Delta S100B and c-TnT were defined as the difference in percentage of increase or decrease compared to the baseline control period (F Δ S100B, M Δ S100B and F Δ c-TnT). During the baseline period, every 20 minutes during each UCO phase and at the end of each UCO phase, a 5-minute period with no UCO was observed to assess fetal hemodynamics, mean arterial blood pressure (Mean ABP), fetal heart rate (FHR), and arterial blood gases. We analyzed the data from the baseline period and from the 5-minute intervals

in between each UCO phases where an additional blood sample was performed for S100B and c-TnT dosages.

Statistics

The gaussian variables are described in terms of mean and standard deviation and non-Gaussian variables in terms of median and interquartile range. The normality of the numerical variables was verified graphically and tested using the Shapiro-Wilk test. Parameter changes during the occlusion phases were evaluated using a mixed linear model with an unstructured covariance structure to account for the correlation between repeated measures and time and baseline data were included as fixed effects. Post-hoc comparisons between the three occlusion phases were performed using linear contrasts. The normality of the residues was verified graphically. In case of normality deviation (without possible log transformation), the comparisons between the three occlusion phases were performed using Wilcoxon's signed-rank test. Bilateral tests were performed with a significance level of 5%. Correlations between biomarker serum levels and acidemia parameters during occlusion phases (at all time of measures) were evaluated using linear mixed models by including sheep as a random effect and time as afixed effect. Statistical analyses were performed using the SAS software (v. 9.4; SAS Institute, Cary, NC, USA).

Ethical approval

The use of anesthesia, surgical procedure, and experimental protocol were consistent with the recommendations of the Ministry of Higher Education and Research. This study was approved by the Animal Experimentation Ethics Committee (Comité d'Ethique en Expérimentation Animale, Avis N°2016121312148878).

131 Results

132 Fourteen ewes were instrumented. Four were excluded from our analysis: two fetuses died in
133 utero at postoperative day 1, one delivery occurred at postoperative day 3 and one occluder
134 ruptured during the first phase of UCO.

135 Ten experiments were performed. Nine ewes had a multiple gestation (Table 1). Five fetuses
136 reached endpoint before the end of the experiment: one during phase B and the other four
137 during phase C.

138 During the baseline control period, all measurements of FHR, mean ABP, and arterial blood
139 gases were in the normal range (Table 2).

140 There was no significant difference between phases A, B, and C for heart rate and mean
141 ABP. There was a significant difference in UCO ABP between phases A and C (54.7 ± 6.2 vs
142 36.1 ±17.2, p = 0.009) and between phases B and C (47.1 ± 15.1 vs 36.1 ±17.2, p = 0.006).
143 Partial pressure of oxygen (PaO_2) was not modified while partial pressure of carbon dioxide
144 ($PaCO_2$) increased significantly between phases A and C (53 mmHg ± 5.1 vs 69.9 mmHg ±
145 16.7, p = 0.006). pH decreased significantly throughout UCO series: 7.29 (7.21-7.37) in
146 phase A vs 7.15 (7.02-7.28) in phase B vs 6.99 (6.86-7.12) in phase C ($p < 0.05$, for all).
147 Lactates increased significantly between the 3 occlusion phases, 5.88 ± 2.35 in phase A vs
148 11.14 ± 3.93 in phase B vs 15.25 ± 1.56 in phase C ($p < 0.05$ for all). Base excess also varied
149 significantly from -1.2 ± 6.3 in phase A to -8.5 ± 7.3 in phase B to -14.8 ± 5 in phase C ($p <$
150 0.05, for all).

151 Serum levels of fetal S100B increased progressively between phases A and B, 11.5 μg ± 3.8
152 vs 12.9 μg ± 4.4, $p < 0.001$. We observed a significant difference in fetal S100B serum
153 concentration when compared to baseline period (ΔS100B), with a progressive increase
154 between phases A and B (7% ± 4 vs 17% ± 13, p = 0.030) and between phases A and C (7%
155 ± 4 vs 24% ± 8, $p < 0.001$).

Fetal c-TnT serum levels increased during occlusions: 102 ng/L (58-119) in phase A, vs 119 ng/L (103-198) in phase B vs 169 ng/L (128-268) in phase C ($p < 0.05$, for all). When compared to the baseline control period, fetal ΔcTnT was significantly modified throughout OCT series: 5.0% (-3; 45) in phase A, 51% (4;263) in phase B, and 77% (56.5; 269) in phase C ($p < 0.05$ for all). There was no difference observed in the maternal S100B and c-TnT dosages throughout the experiment.

Finally, we found a significant correlation between differences in biomarker serum levels throughout the experiment and acidemia parameters, such as pH, lactate, and base excess (Table 3).

Discussion

Main findings

Fetal hypoxia and its early detection during labor remains a major perinatal challenge. The aim is to reduce perinatal asphyxia and its complications, such as HIE. When reproducing a progressive and severe hypoxemia in near-term fetal sheep, we observed a significant rise in fetal S100B and c-TnT serum levels while pH decreases, and lactates rise.

Interpretation

HIE is defined as the clinical manifestation of impaired neonatal brain function following asphyxia due to an antenatal and/or perinatal adverse event (16). Various criteria (biological, clinical and sometimes electrophysiological) are used to define the severity of the neurological damage, many of them included in Sarnats' original classification (16). HIE pathogenesis is fairly complex and is still not understood fully. During HI insult, different intracellular mechanisms are activated, which in turn lead to focal or diffuse cell damage associated with a significant increase in neurobiomarkers, such as S100B (17). S100B is an acid calcium-binding protein specific to the central nervous system where it appears abundant in Schwann cells, glial cells, and neurons. Recently, a series of studies have found that the evaluation of S100B in different body fluids can help early diagnosis of neonates with perinatal asphyxia complicated with HIE (7). In particular, in cord blood samples at birth, S100B was significantly higher in infants with moderate or severe HIE. Increased concentrations were detected 48-72 hours before any clinical, laboratory, or ultrasound signs of brain damage. To our knowledge, the modification of S100B levels during a labor-like acidosis has not yet been evaluated. In our study, we observed an increase in S100B levels during the different phases of the experiment and this increase seems to be detectable as early as the onset of hypoxia, before pH drops below 7.00. In a different model of fetal hypoxia, Giussani et al. reported that, S100B concentrations increased significantly during acute

hypoxemia by 24.9 ± 2.9 % (18). In human studies, the difference in S100B concentrations at birth is statistically significant between neonates with HIE and controls (8,19). The sensitivity of this neurobiomarker could be useful in daily practice as a second-line method for screening fetuses at risk of brain damage. Regarding S100B in maternal blood, an increased concentration has been reported within 3 hours of fetal hypoxic insult. Since no damage was seen in the maternal brain of the animals used, the authors concluded that some of the higher maternal S100B levels were of fetal origin, from across the placental barrier (20). In our study, we did not show any increase in maternal serum levels of S100B in response to severe hypoxia. The possibility of species-specific placental transport of the S100B may explain our differences from the literature.

During labor, several adaptive mechanisms such as heart rate decelerations or tachycardia, allow fetuses to withstand hypoxia (21,22). Following a perinatal HI insult, oxygen deprivation causes myocardial damage and neonates develop cardiovascular dysfunction as part of the multiorgan failure showcasing HIE (23,24). The cTnT concentration is higher in newborns developing HIE compared to those who developed no complications (9). In our study, we found that c-TnT increased early as the pH starts to drop below 7.15. These results reinforce the hypothesis of an anaerobic cardiac metabolism secondary to fetal hypoxia. Similarly, Sweetman et al. showed that neonates with severe neonatal hypoxic ischemia have significantly higher serum c-TnT concentrations than other asphyxiated groups (5). Troponin T may be a reliable marker of myocardial injury and may serve as an adjunct for early diagnosis of perinatal asphyxia.

Strengths and limitations

This study provides data on progressive changes in fetal levels of S100B and c-TnT. There is a clinical interest in understanding how these markers behave during the equivalent of a very challenging labor. However, the small number of experiments and important variability of

results are obstacles to drawing strong conclusions. The baseline state of our fetuses prior to resuming the experiment was not comparable to previous reports, because PaO_2 was low and lactates elevated. We hypothesized that with the breed used and the high rate of multiple gestation, theb fetuses were not comparable to previous studies. The absence of a control group can be discussed, but the biomarker changes were compared to baseline values. Finally, a pH < 6.90 indicated the end of the experiment. Even if models that used pH as a cut off show minimal injury (15,25) compared to those that use hypotension (26), we observed mild to severe hypotension during phase C in our study.

Conclusion

S100B and c-TnT increased when fetal acidosis occurred, reflecting potential neurological damage and fetal cardiovascular adaptation. It will be interesting to correlate those changes to anatomopathology in a larger sample.

227 Competing interests

228 The authors have no conflicts of interest

229

230 Author contributions

231 Y.H., J.D.P, D.S. and L.G. conducted the experiments. Y.H., J.D.P. and L. L collected all
232 data. Y.H. and C.G. wrote the manuscript. E.D. conducted statistical analysis and wrote the
233 statistics methodology. J.D., P.M., V.H.D. and L.S. reviewed and corrected the manuscript.

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313

Ewes	Number of fetuses	Weight of instrumented fetus	Study terminated due to low pH	Phase
1	2	3805	**Yes**	C
2	3	2935	No	
3	2	3555	**Yes**	B
4	2	3850	**Yes**	C
5	2	3135	No	
6	2	3150	**Yes**	C
7	2	3380	No	
8	2	3245	**Yes**	C
9	2	3290	No	
10	1	3125	No	

314

315 **Table 1. Description of our study group**
316 Weights are presented in grams

	Baseline	**Umbilical Cord Occlusions Period**			p		
	S	A	B	C	A vs B	A vs C	B vs C
FHR (bpm)	183.3 ± 10.6	172.2 ± 16.6	181.3 ± 23.1	176.4 ± 22.5	.17	.79	.45
Mean ABP (mmHg)	47.4 ± 10.8	55.3 ± 10.5	58.1 ± 8.0	59.7 ± 9.3	.35	.35	.52
UCO ABP (mmHg)	-	54.7 ± 6.2	47.1 ± 15.1	36.1 ± 17.2	.15	**.009**	**.006**
pH	7.39 ± 0.03	7.29 ± 0.08	7.15 ± 0.13	6.99 ± 0.13	**.001**	**<.001**	**.002**
PaO_2 (mmHg)	13.6 ± 5.5	14.0 ± 3.8	15.2 ± 2.9	16.3 ± 2.7	.12	.078	.17
$PaCO_2$ (mmHg)	46.8 ± 6.2	53.0 ± 5.1	57.2 ± 7.0	69.9 ± 16.7	**.002**	**.006**	**.013**
Base excess (mEq/L)	3.1 ± 4.6	-1.2 ± 6.3	-8.5 ± 7.3	-14.8 ± 5.0	**<.001**	**<.001**	**<.001**
Lactate (mmol/L)	2.51 ± 0.74	5.88 ± 2.35	11.14 ± 3.93	15.25 ± 1.56	**<.001**	**<.001**	**.008**
M S100B (μg/L)	0.44 ± 0.16	0.47 ± 0.14	0.43 ± 0.16	0.45 ± 0.19	.70	.67	.48
M Δ S100B (%)	-	-1.9 ± 2.7	-2.0 ± 5.6	-0.8 ± 0.13	.93	.98	.95
F S100B (μg/L)	10.7 ± 3.4	11.5 ± 3.8	12.7 ± 4.7	12.9 ± 4.4	**.032**	**.001**	.14
F Δ S100B (%)	-	7 ± 4	17 ± 13	24 ± 8	**.030**	**<.001**	.18
M c-TNT (ng/L)[1]	8 (6 ; 14)	8.5 (6 ; 12)	9 (6 ; 14)	10.5 (6 ; 15)	.75	1	1
M Δ c-Tnt (%)	-	6.3 ± 7.9	3.3 ± 11.5	3.5 ± 15.7	.33	.87	.51
F c-TnT (ng/L)[1]	73 (49 ; 105)	102 (58 ; 119)	119 (103 ; 198)	169 (128 ; 268)	**.012**	**.008**	**.008**
F Δ c-TnT (%)[1]	-	5.0 (-3.0 ; 45.0)	51.0 (4.0 ; 263.0)	77.0 (56.5 ; 269.0)	**.012**	**.008**	**.008**

Table 2. Hemodynamic, blood gas and biochemical measures

Data are presented on average ± standard or median deviation (interquartile range).

[1] P-values were calculated using the Wilcoxon signed-rank test, $p < .05$ is significant (given in bold)

FHR: Fetal Heart Rate, bpm: beats per minutes, ABP: Arterial Blood Pressure, F: Fetal, M: Maternal, cTnT: Cardiac Troponin T; UCO: Umbilical Cord Occlusion

	pH		Lactate		Base excess	
F Δ S100B	-0.049 ± 0.16	**0.007**	1.271 ± 0.295	**<0.001**	-2.193 ± 0.559	**0.002**
F Δ c-TnT	-0.001 ± 0.001	**0.034**	0.016 ± 0.005	**0.004**	-0.024 ± 0.009	**0.021**

322

323 Table 3. Correlations between biomarker serum levels with acidemia parameters.

324 Data are presented on β **±** standard error.

325 $p < .05$ is significant (given in bold).

326