The Journal of Maternal-Fetal & Neonatal Medicine

ISSN: 1476-7058 (Print) 1476-4954 (Online) Journal homepage: http://www.tandfonline.com/loi/ijmf20

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To cite this article: Chiara Peila, Alessandra Coscia, Enrico Bertino, Giovanni Li Volti, Fabio Galvano, Gerard H. A. Visser & Diego Gazzolo (2017): Holder pasteurization affects S100B concentrations in human milk, The Journal of Maternal-Fetal & Neonatal Medicine, DOI: 10.1080/14767058.2017.1291618

To link to this article: http://dx.doi.org/10.1080/14767058.2017.1291618

Accepted author version posted online: 05 Feb 2017.
Published online: 28 Feb 2017.

THE JOURNAL OF MATERNAL-FETAL & NEONATAL MEDICINE, 2017
http://dx.doi.org/10.1080/14767058.2017.1291618

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ORIGINAL ARTICLE

Holder pasteurization affects S100B concentrations in human milk

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ABSTRACT

Purpose: Donor milk (DM) represents an important nutrition source for high-risk newborns. Holder pasteurization (HoP) is the most recommended procedure for DM treatment, providing a good compromise between microbiological safety and biological quality. HoP was previously shown to affect DM cytokines, growth factors and hormones levels, whilst no data concerning the possible effects of HoP on neurobiomarkers (NB) are available. Therefore, our study investigated whether the concentration in DM of a well-known NB involved in brain development/damage, namely S100B, changes due to HoP.

Materials and methods: We conducted a pretest-test study in 11 mothers, whose DM samples were sub-divided into two parts: the first was immediately frozen (−80 °C); the second was pasteurized with Holder method before freezing. S100B DM levels were measured using a commercially available immunoluminometric assay.

Results: S100B protein was detected in all milk samples. Results showed significant differences between groups ($p < 0.05$) in S100B levels after HoP.

Conclusions: Our data provide evidence that S100B is present in preterm milk as well as in term milk during maturation degree. Moreover, the results confirm the susceptibility of this neurotrophic factor to pasteurization stresses and the need to develop new storage techniques to preserve the biological quality of human milk.

ARTICLE HISTORY
Received 17 April 2016
Accepted 1 February 2017

KEYWORDS
Central nervous system; donor human milk; human milk; pasteurization holder; S100B

Introduction

Despite technological improvement in milk-formulae milk research and industrial preparation procedures, there are still evidences on the unique composition of breast milk. This issue is of relevance especially in high-risk newborn feeding in terms of multiorgan protection and infections prevention [1]. Therefore, the main target of neonatologists and biochemists points toward a wider use of breast milk in neonatal intensive care units (NICU) and to provide safer procedures for human milk storage and conservation [2]. This latter point is noteworthy since milk stored in Human Milk Banks (HMB) after pasteurization may present potential qualitative/quantitative changes in its composition [3]. Thus, the optimal pasteurization process should be able to provide as much as possible a safe and unaltered milk composition [3]. To date, Holder pasteurization (HoP) (62.5 °C for 30 min) is the most studied and recommended method for the heat treatment of donor human milk (DM) [2]. HoP provides a good compromise between microbiological safety and nutritional and biological quality of the human milk. Nonetheless, HoP inactivates or reduces some immunologic and anti-infective factors [4–6], while data on its effects on other constituents are unknown or still matter of investigation. This especially holds for neurobiomarkers (NB) such as neurotrophic factors and calcium binding proteins, including S100B protein, known to play a relevant role in brain development [7,8].

S100B is an acidic calcium-binding protein, mainly concentrated in the central nervous system (CNS) and detectable in a variety of biological fluids including milk, where it is concentrated 200–300 fold higher than in other fluids [9,10]. Based on the protein's main known activities (neurotrophic, brain damage marker), S100B regulates different cellular functions such as growth, intercellular communication, cellular transduction signal, and cellular metabolism. Bearing in mind the unique properties of breast milk, S100B has been investigated both in human and in milk-formulae milks [11].

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Table 1. Demographic characteristics of milk donors.

Parameters	$n = 11$
Mean maternal age, years	35
Parity 1, *n* (total)	7 (11)
Mode of delivery, *n* (%)	
Caesarean	4 (35)
Vaginal	7 (65)
Gestational age, weeks	36
Mean birth weight, g	2950
Apgar score >7, *n* (total)	
At 1 min	9 (11)
At 5 min	10 (11)
Gender male (female)	5 (6)

Results showed that S100B levels (i) in maternal milk were higher than formulae-milks and cow-milk [11]; (ii) correlated with the degree of milk maturation [11]; (iii) in formulae milks were affected by industrial preparation such as pasteurization and spray-drying procedures [12]. In this regard, data on potential effects of HoP procedure on S100B levels in human milk are still lacking.

Therefore, in the present study, we investigated the effects of HoP on S100B milk concentrations from healthy mother donors.

Materials and methods

We conducted a pre-test/test study, where the milk donors acted as their own controls. Milk samples (colostrum: $n = 11$; transition: $n = 11$; mature: $n = 11$, defined according to Playford et al. [13]) were collected from 11 healthy mothers from consecutive singleton physiological pregnancies (Table 1).

Exclusion criteria were multiple pregnancies, gestational diabetes, and any maternal CNS illness; pregnancies carrying a fetus with any malformation and/or chromosomal abnormalities, systemic infection, intrauterine growth retardation, or cardiac or hemolytic disease; mothers who were tobacco smokers, drugs, and alcoholic addicted; use of drugs or pharmacologically active substances; mothers who received blood transfusions or blood products, or organ transplants; perinatal asphyxia and dystocia.

The study protocol was approved by the local Ethic Committee of the Italian Association of Human Milk Donor Banks. Mothers admitted into the study gave signed and informed consent.

Collection and pasteurization of human milk

Fresh milk samples were collected at the same timepoint (9–10 am) into sterile, disposable, high-density polyethylene-sealed bottles (Flormed, Napoli, Italy). Milk was obtained by emptying one breast completely by means of an electric breast pump (Medela Symphony, Baar, Switzerland). From the total amount of milk of each mother, a sample of 10 mL of milk was collected and then subdivided into two aliquots. The first was immediately frozen at −80 °C (NO-HoP), while the second aliquot was pasteurized and then frozen at −80 °C (HoP).

The HoP was performed with a Sterifeed pasteurizer (Medicare Colgate Ltd, Cullompton, UK) heating milk samples at 62.5 °C for 30 min, then cooling to 10 °C in approximately 20 min by immersion into cold water.

S100B measurements

Samples were immediately stored at −80 °C until measurement. The S100B was measured in all samples using a commercially available immunoluminometric assay (Liaison S100, DiaSorin, Saluggia, Italy) according to the instructions from the manufacturer. Investigators who performed the laboratory tests were blind to storing modalities. The assay detection limit was 0.02 μg/L, the intra-assay CV was ≤5.0%, and the inter-assay CV was ≤10%. The assay is specific for S100B, having been assessed by the manufacturer for a lack of cross reactivity with other proteins of the S100 family.

Statistical analysis

S100B concentrations are expressed as median and interquartile ranges. Statistical analysis was performed by a comparison between groups using Mann–Whitney two-sided *U*-test when the data did not follow a Gaussian distribution. A $p < 0.05$ was considered significant.

Results

Maternal and perinatal characteristics of the milk donors are reported in Table 1. All mothers showed normal clinical conditions. No overt neurological injury and/or infections were observed at the sampling timepoints or at discharge from the hospital.

S100B protein was detectable in all the measured milk samples. No significant differences ($p > 0.05$, for all) have been found in S100B concentration when corrected for the degree of milk maturation. Moreover, S100B levels did not differ ($p > 0.05$) between samples collected from mothers of preterm (median: 69.44 μg/L; 25° centile: 53.79 μg/L; 75° centile: 71.66 μg/L) and term (median: 75.55 μg/L; 25° centile: 58.83 μg/L;

Table 2 S100B levels (μg/L) in milk in not pasteurized (NO-HoP) and pasteurized (HoP) groups. Data are expressed as median and interquartile ranges.

	All samples ($n = 33$)			Colostrum ($n = 11$)			Transition ($n = 11$)			Mature ($n = 11$)		
Parameters	Median	25%	75%	Median	25%	75%	Median	25%	75%	Median	25%	75%
NO-HoP	129.50	96.30	164.0	129.50	86.10	163.0	164.00	109.37	173.75	111.0	74.72	125.75
HoP	70.00	41.60	109.0	72.80	26.40	110.0	83.40	70.00	110.50	41.60	15.92	68.9

75° centile: 83.70 μg/L) infants after correction for gestational age.

In Table 2, S100B levels before and after HoP procedure are reported. Significant differences in S100B concentrations were observed in all milk samples before HoP and after HoP process ($p < 0.05$). After correction for milk maturation degree, we observed a significant reduction after HoP in transitional milk and mature milk ($p < 0.05$) but a no significant difference in colostrum after heat treatment ($p > 0.05$).

Discussion

Nowadays, mother milk still represents the first feeding choice for all neonates. This unique nutrient is of the utmost importance especially for sick children, who require mother milk to reduce the risk of complications related to prematurity such as necrotizing enterocolitis, sepsis, and bronchopulmonary dysplasia [1]. In this respect, DM was shown to represent a valid alternative [3], thus supporting the need of HMB. Of note, HMB guidelines require that DM has to be pasteurized prior to use to inactivate viral and bacterial agents [2,13,14]. Heat treatment has been reported to affect, at least in part, the nutritional and immunological properties of human milk [4–6], although no information are available to date concerning the potential effects of HoP on human milk CNS constituents.

The present study shows that HoP procedure affects the concentration in milk of a well-established CNS constituent, namely the S100B protein. The difference in protein concentration regarded transitional and mature milks, while no changes due to HoP procedure have been found in colostrum. Moreover, no differences among different degrees of milk maturation have been observed. The discrepancy with previous observations [9,11] may reside in different studied populations (term versus preterm-term).

S100B levels detected in milk were considerably higher when compared with those of other biological fluids [9,10]. This latter finding is in agreement with previous observations [11] and is consistent with the notion that calcium binding proteins are highly concentrated in a biological fluid such as milk in which calcium is abundant [9,10,15].

The finding of different S100B concentrations before and after HoP procedure constitutes the first observation on the effects of the pasteurization on the protein and deserves further consideration. In particular S100B has been shown to be (i) thermostable both at room temperature and after freezing from samples collected in different biological fluids [16–18]; (ii) affected, in artificial milk, by techniques in use for industrial procedures, such as spray-drying (180–185 °C) [12], and (iii) stable at pasteurization procedure applied in industrial procedures for treatment of milk formulae (70–72 °C for 5–15 s) [12]. Altogether, bearing in mind that the HoP procedure consists of a heat treatment at 62.5° for 30 min, the possibility that medium-low temperature but for longer time than industrial procedures could affect S100B is consistent. Another explanation for the lower levels of S100B in DM may reside in the possibility that the epitopes of the protein have been modified during HoP, limiting the accuracy in the quantitative protein measurement. In addition, the possibility that HoP could also affect S100B reducing or destroying its biological activity has to be taken into the due account. The finding is of relevance since HoP has been previously shown to reduce the proteins, cytokines, growth factors, and hormones content of milk [4–6]. Further studies on a wider study population are thus needed in order to optimize CNS constituents' measurement in milks as well as to improve DM components stability during treatment and storage processes.

The highest S100B levels pre-HoP and their decrease in milk concentrations following HoP warrant further consideration. The former issue offers additional support concerning the neurotrophic role of this NB [19]. In this regard, there is evidence both in humans and in animal models on the trophic role of the protein in CNS fetal/neonatal development. In detail, S100B has been shown to (i) stimulate neurite outgrowth [20] through a cascade of events in which nuclear translocation of NF-kB, up-regulation of Bcl-2 in neurons, and receptor for advanced glycation end products (RAGE) are involved [21,22], (ii) exert a protective action on neurons, through a RAGE-mediated

effect and the activation of the Cdc42/Rac signaling pathway, under physiological (development) and pathological conditions [23,24], (iii) enhance hippocampal neurogenesis, and to prevent motor neuron degeneration after sciatic nerve section [25,26], (iv) be involved in the mechanisms of modulating learning and memory [27], and (v) be involved (in mice pups of immunized mothers that did not express S100B protein in their milk) in morphological problems, a delay of the maturation of neurobehavioral systems and a deficit of neuromotor functions (i.e. visual abilities, somato-sensory and posture reactions, muscular strength, locomotion, and fear/orienting processes) [28]. Altogether, the evidence that milk concentration of a brain constituent involved in CNS development and protection is affected by HoP strengthens the notion on the need of further studies aimed at limiting DM depletion thus empowering its unique role [3]. This issue can be of relevance especially for the feeding of high-risk newborns. Further studies on a wider study-population are needed in order to corroborate S100B transfer from gut to systemic circulation. In this regard, it has been recently observed that in the human gut, S100B protein is specifically and physiologically expressed and released in the gut by enteric glial cells, morphological and functional equivalent of astrocytes and microglia in the CNS. The S100B protein can be considered as an easily diffusible pro-inflammatory cytokine which gains access to the extracellular space especially during immune inflammatory reactions [29].

In conclusion, the present data suggest that HoP significantly modifies the concentration of S100B. This finding opens up to further investigations on NBs assessment in human milk and their pre-analytical stability according to storage procedure.

Disclosure statement

The authors report no conflicts of interest.

Funding

This study is part of the I.O. PhD International Program, under the auspices of the Italian Society of Neonatology and of the Neonatal Clinical Biochemistry Research Group, and was partially supported by grants to DG from I Colori della Vita Foundation, Italy.

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