Research Article

S100B Protein concentration in milk-formulas for preterm and term infants Correlation with industrial preparation procedures

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Human milk S100B protein possesses important neurotrophic properties. However, in some conditions human milk is substituted by milk formulas. The aims of the present study were: to assess S100B concentrations in milk formulas, to verify any differences in S100B levels between preterm and term infant formulas and to evaluate the impact of industrial preparation at predetermined phases on S100B content. Two different set of samples were tested: (i) commercial preterm (n = 36) and term (n = 36) infant milk formulas; ii) milk preterm (n = 10) and term infant (n = 10) formulas sampled at the following predetermined industrial preparation time points: skimmed cow milk (Time 0); after protein sources supplementation (Time 1); after pasteurization (Time 2); after spray-drying (Time 3). Our results showed that S100B concentration in preterm formulas were higher than in term ones ($p < 0.01$). In addition, S100B concentrations during industrial preparation showed a significant increase ($p < 0.001$) at Time 1 followed by a slight decrease ($p > 0.05$) at Time 2, whereas a significant ($p < 0.001$) dip was observed at Time 3. In conclusion, S100B showed a sufficient thermostability to resist pasteurization but not spry-drying. New feeding strategies in preterm and term infants are therefore warranted in order to preserve S100B protein during industrial preparation.

Keywords: Formulas / Human milk / Industrial preparation / S100B / Thermostability

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1 Introduction

S100B is an acidic calcium-binding protein characterized by the presence of a pair of so-called EF-hand (*i.e.* helix-loop-helix) calcium-binding motifs, first discovered in the crystal structure of parvolbumin, which induce conformational changes of the protein after binding to calcium [1]. First isolated in 1965 by Moore [2] from the nervous system, it is highly concentrated in glial cells and in specific neuronal populations [3]. Among the pleiotropic S100B functions, a neurotrophic effect has been reported as well as its ability to regulate different cellular functions such as growth, intercellular communication, cellular transduction signal and cellular metabolism [4]. The protein is known to be detectable at physiological concentration in different biological fluids including human milk in which it is concentrated 200 – 300 fold higher than other fluids [5]. The explanation resides in the protein's trophic role in biological fluids, such as milk, that is believed to contain several biological factors involved in the regulation of newborn growth and brain development [6, 7]. In this regard, it has been reported that preterm and term infants fed by breast milk have faster brainstem maturation [8, 9], compared with infants fed formula. The explanation resides in the unique

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composition of breast milk, although the impact of exogenous confounding factors such as parental socio-economic background have to be taken into consideration, and the possibility that industrial procedure routinely employed to produce milk formulas could somewhat affect cow milk nutritional properties [10]. The hypothesis is consistent with different S100B concentration between human and milk formulas previously reported [11]. However, data on the fate of S100B during manufacture processes of milk formulas for preterm or term infants, such as blending, pasteurization, homogenization, sterilization and spray-drying procedures are to date lacking.

Therefore, the aim of the present study was to investigate whether S100B concentration in milk formulas: (i) differ between preterm and term infants; (ii) vary in accordance to the productive process as assessed by a longitudinal measurement of the protein at predetermined production phases.

2 Materials and methods

2.1 Preterm and term milk formula samples

From September 2006 to May 2007 we assessed the concentration of S100B protein in commercially available preterm (n = 36) and term (n = 36) infant milk formulas provided from different manufacturers.

All samples were anonymous and progressively numbered, and were analyzed by the same operator who was aware of neither milk provenience nor features.

2.2 Industrial preparation samples

From March to May 2007 we assessed the concentration of S100B protein in samples, from milk formulas dedicated to preterm (n = 10) and term infants (n = 10), obtained at the following predetermined manufacture time points. Figure 1 reports a simplified flow-chart of formula industrial preparation with S100B monitoring timepoints as follows: Time 0: skimmed cow milk, before supplementation with protein and lipid sources; Time 1: after supplementation with protein and lipid sources, before pasteurization; Time 2: after pasteurization before spray-drying; Time 3: after spray-drying procedure.

The study protocol was approved by the local Ethics Committees of the Institutions participating to the investigation.

2.3 S100B measurements

S100B protein concentration was measured in all samples using a specific immunoluminometric assay (LIAISON S100B, Dietzenbach-Germany). According to the manufacturer's indications, this assay is specific for the β subunit of the protein as defined by the three monoclonal antibodies SMST 12, SMSK 25 and SMSK 28. The β subunit of the S100 protein is known to be predominant (80 – 96%) in the human brain [12, 13].

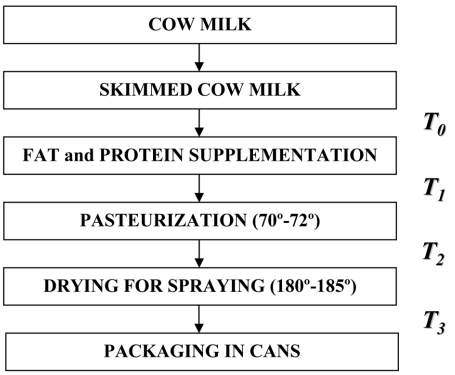

Figure 1. Simplified industrial preparation flow-chart and S100B assessment timepoints. Time 0: skimmed cow milk, before supplementation with protein and lipid sources; Time 1: after supplementation with protein and lipid sources, before pasteurization; Time 2: after pasteurization before spray-drying; Time 3: after spray-drying procedure, respectively.

Each measurement was performed in duplicate and the averages were reported. The LOD of the assay (minimum measurable S100B value: B0 value ±3 SD) was 0.02 μg/L. The precision, CV, was < 5% for the intra-assay and < 10% for the inter-assay.

2.4 Statistical analysis

S100B concentrations in milk formulas are expressed as median (5 – 95th centiles) and in percentage of change from Time 0 (defatted cow milk, before supplementation with protein and lipid sources). Comparison between groups was performed by Mann – Whitney U test when values were not normally distributed. Comparison among different monitoring time points was analyzed by Kruskal – Wallis one-way ANOVA. Statistical significance was set at $p < 0.05$.

3 Results

S100B protein was detectable in all samples examined. In particular, S100B concentration in milk formulas for preterm infants (median 4.18 μg/L; 5th centile: 0.90 μg/L; 95th centile 22.7 μg/L) were significantly higher ($p < 0.01$) than those for term (median 2.65 μg/L; 5th centile: 0.68 μg/L; 95th centile 9.28 μg/L).

S100B pattern during industrial preparation was characterized, both in preterm and term milk preparations, by a significant increase ($p < 0.001$) in protein concentration peaking at Time 1 (after supplementation with protein and lipid sources), followed by a slight decrease ($p > 0.05$) at Time 2 (after pasteurization before spray-drying) and a significant ($p < 0.001$) dip at Time 3 (after spray-drying proce-

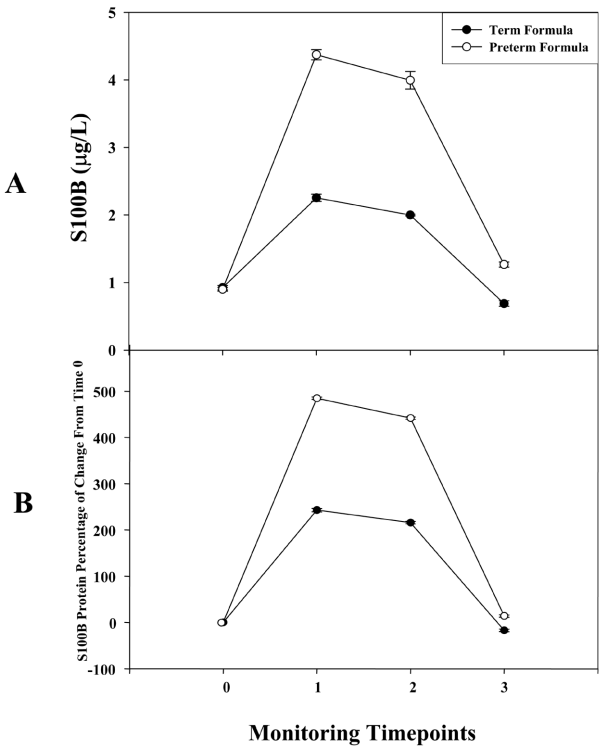

Figure 2. S100B protein concentrations espressed expressed as μg/L (Panel A) and as percentage of change (Panel B) at different industrial preparation procedures (Time 0: skimmed cow milk, before supplementation with protein and lipid sources; Time 1: after supplementation with protein and lipid sources, before pasteurization; Time 2: after pasteurization before spray-drying; Time 3: after spray-drying). Values are expressed as median and 5 – 95th centile, respectively.

dure) (Fig. 2). No statistical differences ($p > 0.05$, for both) have been shown between Time 0 and 3 in both groups.

S100B concentrations during industrial preparation, expressed as absolute values or as percentage of change respect to Time 0, did not differ between preterm and term products at time 0 ($p > 0.05$) whereas they were significantly higher ($p < 0.01$, for all) in preterm than term at time points 1, 2 and 3. No significant differences were observed between Time 0 and Time 3 in the two formulas when compared for absolute values and in percentage of change ($p > 0.05$).

4 Discussion

The present study provides evidence that S100B protein is present in milk formulas at different concentrations in preterm and term milks. There is evidence that the significant changes in S100B milk concentrations in different formulas occurred during industrial preparation procedure. In particular, the supplementation with protein source and spray-drying procedures constitute the main industrial phases involved in the enrichment and in the decrease in protein concentration in milk formulas, respectively.

The finding constitutes the first observation on the potential impact of industrial preparation procedure on S100B protein in formulas. However, despite the novelty and originality of our observations, the present data are not surprising, since it has been previously reported that these processes can somehow affect the overall protein content and/or structure [10]. In this respect, the analysis of the protein pattern at predetermined monitoring time points during industrial preparation showed that, before supplementation with protein sources, S100B concentrations in defatted cow milk were superimposable in both preterm and term milk formulas. The finding is consistent with previous observations and confirms the reliability of the employed measurement method [11, 14].

The remarkable increase in S100B concentration observed in formulas at Time 1 (after the supplementation phase) is due to enrichment (*i. e.* milk whey) that usually occurs at this stage and therefore difference in S100B concentration between formulas is attributable to different quantitative addition of proteins. It is noteworthy that at Time 1 S100B concentration, in preterm formulas, was comparable to that found in human milk [11] (after correction for preparation modalities, *i. e.* milk dilution).

A fast decay of protein concentration in milk formulas was observed starting from pasteurization (Time 2) to spray-drying procedure (Time 3): pasteurization*, per se,* seems to have a mild effect in S100B decrease probably due to protein thermostability. In this respect, it has been shown that S100B is stable at room temperature up to 120 h after refreshment [15] and, therefore, the possibility that S100B could be sufficiently stable during pasteurization is a fact that has to be taken into due account. Conversely, the fast decay in S100B concentrations after spray-drying procedures warrants consideration. The explanation resides in high temperature (about 180°C) side-effects on S100B protein occurring during this procedure: the spray-drying procedure likely causes a heat induced denaturation [10] with substantial modifications of protein epitopes that might modify its biological activity. Finally, we can not exclude that during formula preparation diverse reactions can occur between proteins and other nutrients, *i. e.* glycosilation, Maillard reactions or other non-enzymatic Browning reactions [10, 16, 17].

The evidence that a brain constituent, such as S100B, is present at such lower concentration in formulas when compared with human and cow milk warrants consideration. Bearing in mind that: (i) infant commercial formula composition differ significantly from human milk [11]; (ii) the unique composition of breast milk; iii) infants fed breast milk have faster brainstem maturation and neurodevelopmental outcome [6 – 9, 18, 19]; all together, the present findings suggest the need to improve the composition of formulas in order to minimize the gap with human milk and

S100B should be one of the nutritional factors worth to be added. Indeed, a series of evidences indicate that S100B acts as a cytokine with a neurotrophic effect during both development and nerve regeneration. In particular, it has been reported that S100B: (i) stimulates neurite outgrowth [20] triggering a cascade of events involving nuclear translocation of NF-*k*B, up-regulation of Bcl-2 in target neurons and finally the binding of the protein to RAGE, Receptor for Advanced Glycation End Products, [21, 22]; (ii) enhances survival of neurons during development (through a RAGE-mediated effect and the activation of the Cdc42/Rac signalling pathway) [23, 24] and after injury (intraventricular S100B infusion enhances hippocampal neurogenesis in rats) [25]; (iii) prevents motor neuron degeneration in newborn rats after sciatic nerve section [26], (iv) is involved in the mechanisms modulating learning and memory [27, 28].

Taken together, molecular, experimental and clinical findings provide evidence on the opportunity to supplement milk formula with S100B protein and open-up a new clue on speculation about new feeding strategies in preterm and term infants. Further investigations on the possibility to preserve S100B protein and other proteins during industrial preparation are now warranted.

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The authors have declared no conflict of interest.

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