

Effects of pasteurization and refrigerated storage on human milk neurobiomarkers concentrations

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Chapter 7

Summary & conclusions

Summary and Discussion

Recently, several studies have emphasized that fresh own mother's breast milk is the first choice in preterm infant feeding and that strong efforts should be made to promote lactation [1]. For this reason, it has been the goal of international neonatal networks to increase breastfeeding rates at discharge from the NICU above the threshold of >20%. Nonetheless, when mother's own milk is not available, DM is highly recommended [1]. DM should be provided from an established HMB, which has to follow specific safety guidelines [2]. The presence of a HMB does not decrease breast-feeding rates at discharge, but decreases the use of formula during the first weeks of life [3].

Among the reported evidence of the benefits in preterm newborns deriving from the use of DM, protection against NEC is particularly important and should be highlighted [4]. The potential advantages of unfortified DM on improved feeding tolerance and a reduced cardiovascular risk during adolescence are still a matter of debate [3].

Storage and processing of HM reduce some of its biological components, which may lose, in part, some of their health benefits. From a nutritional point of view, DM (like HM) is constantly subject of research since new molecules involved in developmental processes have been identified, such as the "*trophic factors*" [5].

Future research should focus on improvements of milk processing in HMB (particularly heat treatment) and on further evaluation of the potential clinical benefits of processed and fortified DM.

In **Chapter 1** we have provided an overview of the main findings related to the effects of HoP on the biological and nutritive components of DM and have shown a high variability among literature data. Apparently it is very difficult to quantify the actual effects of HoP on the biological and nutritional properties of the milk. Substantial discrepancies exist not only between the protocols applied in different studies but also between the standard operating procedures adopted by HMBs and the experimental methods reported in research protocols. When pasteurization is performed for research purposes, it is usually carried out with laboratory-scale equipment, which is not equivalent to the pasteurization environment used in HMBs [6,7]. Moreover, samples submitted to thermal treatment in laboratories often consist of a few milliliters of milk (typically less than 10 mL) [7,8], whereas HMBs routinely use industrial pasteurizers, specifically designed and validated for the pasteurization of larger quantities of human milk (up to 200 mL of milk for each container). Another limitation is the fact that several studies do not provide precise information concerning the equipment used and the pasteurization parameters applied in the study (e.g., time, temperature, and duration of the different phases of the processing) [9,10]. Furthermore, conditions applied in research protocols for milk expression, handling, processing and storage before analysis are extremely variable, whereas storage conditions in HMBs are standardized and well defined by written pro-

tocols [6-13]. Based on these considerations, it is not surprising that significant differences are present when comparing results obtained in clinical practice and research conditions. Thus, it remains a matter of debate whether the studies available in literature adequately describe the impact of HoP on milk properties and if the nutritional qualities of milk are preserved after pasteurization.

Altogether, the available data show that HoP affects several HM components, even though it is rather difficult to quantify the degradation level. Additionally, it appears that some biochemical patterns have been investigated more extensively than others, whereas some milk components were not considered at all. Proteins are more significantly affected by HoP but the results concerning specific biologically active molecules (such as cytokines and growth factors) remain uncertain, due to the vast number of different compounds analyzed in each study and to the paucity of comparable results.

Our studies were designed to investigate HoP adapting the protocols to match the actual procedures of storage, handling and processing of donor milk practiced by HMBs, to provide results relevant for the actual clinical management of DM.

In **Chapter 2** we evaluated the effect of HoP on the protein profile using a semi-quantitative GeLC-MS analysis technique. GeLC-MS was chosen as it is particularly well-suited to evaluate the complex array of biologically active proteins composing the HM protein profile. Samples obtained from different donors reacted differently to heat treatment although processed and stored at the same conditions. Briefly, about 30% of the samples analyzed showed differences in the protein profile after HoP, whereas the remaining 70% did not. The main detectable protein profile changes were observed in colostrum, thus providing further support to the use of DM since no evident changes were shown in mature milk. These findings deserve further investigation. Specifically, the main open question concerns the interactions between proteins and sugars or lipids due to thermic treatments, with the possible generation of inactive (or even toxic) derivatives.

In **Chapter 3** we investigated the effects of HoP on the DM concentration of a well-established biomarker of CNS development/damage, namely Activin A. Results showed that HoP does not affect Activin A concentrations in DM at different milk maturation degrees. Furthermore, the present study showed first the presence of Activin A in milk collected from preterm deliveries. The loss of biological factors through pasteurization may have significant implications; this is particularly important in preterm infants with an immature immune system, who are at increased risk of developing NEC. The evidence that HoP guarantees an unaltered Activin A intake to the newborn further confirms the benefits of feeding preterm newborns with DM as compared to artificial milk [14]. This is of relevance considering that artificial milk industrial preparation procedures have been shown to affect to a significant extent both the milk composition and properties [15]. Moreover, an unaffected Activin A concentration in DM is important since on the basis of several studies it is reasonable to assume that Activin A is involved

in embryogenesis and exerts a unique trophic effect in biological fluids [16]. In fact, Activin A, its receptors and binding proteins are widely distributed throughout the brain [16]. Studies in experimental models and in case of acute brain injury in the human strongly correlate enhanced Activin A expression as a common response to acute neuronal damage of various origins. Even interesting is the finding that Activin A is able to support the survival of neurogenic cell lines and neurons and to offer protection against neurotoxic damage [17]. Moreover up-regulation of this neurotrophic factor by antidepressant treatment and atrophy of limbic brain regions in response to stress or in depressed patients has resulted in a neurotrophic hypothesis of depression [18]

HM is believed to contain biological factors involved in the regulation of newborn growth, including brain development. Moreover the presence of S100B, a calcium-binding protein in a biological fluid such as milk, in which calcium is abundant, is not surprising in the light of the consideration that other calcium-binding proteins (e.g. alpha-lactalbumin, calmodulin, osteocalcin) have already been detected in milk [19-21].

Therefore, in **Chapter 4** we investigated whether HoP procedure could somehow affect the neurotrophic S100B protein. Results showed an “unexpected” susceptibility of the protein to HoP, especially in transitional and mature milks, whereas no differences were detectable in colostrum. These findings deserve further consideration. The available data confirm that i) S100B collected from different biological fluids is stable both at low and room temperature for long time [19,22], ii) industrial pasteurization procedures for treatment of milk formulae (70-72°C for 5-15 seconds) do not affect S100B [15], and iii) S100B is affected by industrial spray-drying techniques (180-185°C) [15]. Overall it is possible to conclude that temperature *per se* does not affect S100B characteristics. Nonetheless, bearing in mind that the HoP procedure consists of a heat treatment at 62.5° for 30 minutes, the possibility that medium-low temperature for longer time could affect S100B is quite well possible. Another explanation for the lower levels of S100B in DM may reside in the possibility that the epitopes of the protein are modified during HoP, limiting the accuracy in the quantitative protein measurement. In addition, the possibility that HoP could also affect S100B by reducing or destroying its biological activity has to be taken into account.

In **Chapter 5** we studied the effects of HoP on heat shock proteins (HSPs) such as HO-1. Among the several functions of HM, antioxidant properties have also been attributed [23]: the explanation may reside in the presence of antioxidant enzymes and HSPs that have been related to the capacity of binding to specific cell surface receptors such as CD91 [24]. CD91 is an important immunoregulatory receptor that has been previously identified as a receptor for the serum protein α 2-macroglobulin (a ‘natural’ protease inhibitor) and then as a common receptor for all identified HSPs [25,26]. An increasing body of evidence suggests that CD91 represents an important route for stimulating a CD8+ T-cell response by major-histocompatibility-complex-class-I-restricted presenta-

tion. This may also be a fairly general mechanism by which the innate immune system may stimulate the adaptive arm in viral infections and tumors [27].

Among HSPs, HO-1 is the rate-limiting enzyme in heme catabolism that is associated with strong protective effects [28]. The presence of HO-1 has been recently detected in biological fluids (blood, cerebrospinal fluid, and milk), where it is likely involved in several cytoprotective actions. In milk, HO-1 should be also involved in the development and protection of the gastro-enteric tract [29,30]. In particular milk HO-1, similarly to other milk antioxidant enzymes, may exert protection via its antioxidant activity and HO-1 has probably an immunoregulatory role related to its ability to bind specific receptors, such as antigenic peptides and chaperokines.

Our study showed that HO-1 is present in preterm HM and in DM. No statistically significant differences were found between preterm and term milk samples; likewise, our results showed a non-significant decrease in HO-1 levels as the milk matures, in both term and preterm milk. Moreover, our data showed that this heat treatment does not affect HO-1 concentrations also after correction for gestational age and maturation degree of the human milk.

Bearing in mind the different functions of HO-1 in several tissues and specifically in development and regulation of immune system of the gastro-enteric tract, it is evident that HO-1 is unaffected by heat treatment. Its thermostability confirms the biological value of HM associated with the HO-1 content in preterm babies fed with DM.

Finally, in **Chapter 6** we evaluated the effect of cold storage on AM concentration. Our results showed for the first time that AM levels are: i) detectable in HM, ii) higher in HM of preterm infants, and iii) affected by freezing procedure.

The first issue is of relevance due to AM localization of AM especially in the cardiovascular and central nervous system. Taking into account that AM is synthesized in the mammary gland, it is reasonable to assume that its site of releasing and concentration could only be breast milk [31]. However, further investigations performing western-blot analysis and RT-PCR will elucidate whether AM measured in HM corresponds to that currently measured in different biological fluids [32-34].

The second issue offers additional support to the gastrointestinal tract localization of the peptide [35], to its differences in concentration according to different milk maturation degrees and between healthy mothers and those with diseases [36-38]. Altogether it has been suggested that AM may have an important role in the regulation of secretory-motor functions growth and maturation of the neonatal gastrointestinal tract, as well as in its development during the embryogenesis.

Lastly, the third point showed that AM levels are affected by storage procedures decreasing AM levels, at 96-h from storage, up to 98% of the total peptide amount [39-41]. Among different AM functions it has also been reported to act as an antimicrobial agent in the gastrointestinal tract [42,43]. Thus, the finding of higher AM levels detected in HM of preterm infants suggests a trophic role of the peptide at this period. These

data are in agreement with other studies showing that different neuro-biomarker concentrations (i.e. activin A and S100B) are gestational age dependent [44,45]. The fact is of relevance in terms of different vascular, neuronal arborization and immune system maturation and higher occurrence of NEC. Thus future perspectives should regard not only the optimization/creation of storage procedures but also technological improvement in biomarkers assessment in a complex biological fluid such as milk.

Conclusions and Future Perspectives

The present thesis contributes to a better understanding of the potential effect of storage on HM and primarily on the effects of HoP on DM. However, many questions remain to be answered. In particular, future studies must be aimed at improving the biological quality and safety of DM and should be: i) designed to investigate the pre-analytical stability of these components according to the storage procedures; ii) intended to evaluate innovative test technologies, such as metabolomics; iii) focused on new pasteurization techniques (high-temperature short-term pasteurization, thermos-ultrasonic treatment, high-pressure processing, and Ohmic heat treatment); iv) aimed to evaluate analytical techniques able to assess the protein changes due to thermic treatments, as well as their interaction with sugars and lipids; v) designed to evaluate the effects of HoP on other biomarkers involved in growth and developing of newborns.

Moreover our findings open up to further investigations aimed at elucidating the protein stability during industrial processes for the preparation of artificial milk such as pasteurization and spray-drying, which have already been shown to affect milk composition and properties.

Further data concerning the metabolic fate of the most important milk biomarkers in the gastro-enteric tract is needed to corroborate the hypothesis that these participate in the nutritional effects of milk and in its immuno-regulatory and trophic role for intestine and brain development. In this setting, neuro and calcium binding proteins, vasoactive agents, oxidative stress markers are involved in the known (patho-) physiological steps in multiorgan growth. The possibility to evaluate the concentrations of these elements in DM or in artificial milk will be the first step to elaborate specific therapeutic strategies in selected newborns. This means that biomarkers are of potential relevance to provide useful supplementation in future therapeutic protocols, being able to empower neuro/enteral-protection at different timing and length of administrations.

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