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Heme oxygenase-1 in donor human milk.

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Abstract

Background: When mother's own milk is not available, the best alternative is represented by donor milk (DM), i.e., human milk pasteurized with the Holder pasteurization (HoP) method in Human Milk Banks for safe storage. Advantages and disadvantages associated to this procedure have been widely discussed in Literature but currently represent the best compromise between microbiological safety and biological quality of DM.

Objective: The aim of this study is to investigate the effects of HoP on Heme-oxygenase-1 (HO-1), an antioxidant protein involved in several cytoprotective actions that should play an important role in the development and protection of the gastro-enteric tract.

Methods: We collected 42 milk samples and we performed a pretest-test study where the milk donors acted as their own controls in 14 mothers (who have delivered 7 at term and 7 preterm of Gestational Age). Milk samples were divided into two parts: the first was frozen (-80°C); the second was Holder-pasteurized before freezing (-80°C). HO-1 was quantified using an ELISA test.

Results: HO-1 was detected in all samples. There were no significant differences in HO-1 concentrations between term and preterm milk samples (P>0.05). Likewise, no significant differences in HO-1 content were found between raw and pasteurized milk samples (P>0.05). There were no significant differences when studied groups were corrected for milk maturation degree (P>0.05).

Conclusion: The data suggest that HO-1 does not significantly differ between preterm and term milk samples and that HoP does not affect HO-1 concentration in human milk.

Keywords: Human milk, Donor human milk, Pasteurization holder, Hemeoxygenase-1, Nutrition of preterm infants, NEC.

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Background

In literature, there are several evidences of the unique role of human milk for infant nutrition [1,2]. Feeding preterm and term infants with human milk is associated with significant long-term beneficial effects such as reduced risk of cardiovascular diseases, diabetes, obesity and cancer [1,3]. During the first months of life, short-term benefits are also present, such as supporting the development of the immune system and providing protection against infectious diseases [1]. When mother's own milk is not available, the best alternative is represented by donor milk (DM), i.e., human milk pasteurized with the Holder Pasteurization (HoP) method in Human Milk Banks (HMBs) for safe storage [4,5]. The available literature confirms the persistence of some of the benefits of human milk also in donor milk, especially regarding the protection against Necrotising Enterocolitis (NEC) for preterm newborns [6-9]. The short term benefit of HM and DM is supported probably not only by secretory immunoglobulin A but also by other immunologically active components such as antimicrobial factors, cytokines, chemokines and growth factors [10-13]. Additionally, the antioxidant properties

attributed to HM play a role of paramount interest, although the underlying mechanisms are still unclear [14,15]. The explanation may reside in the presence of antioxidant enzymes, an important group of which is constituted by Heat Shock Proteins (HSPs), whose presence in biological fluids has been associated to the capacity of binding to specific cell surface receptors such as CD91 [16]. Among HSPs, heme oxygenase-1 (HO-1 or HSP32) is a stress-inducible rate-limiting enzyme in heme-catabolism which is associated to a strong cytoprotective effect thanks to its multiple catalytic by-products [17]. HO-1 has recently been detected in different biological fluids (blood and cerebrospinal fluid), where its presence is reasonably related to its putative protective action [18,19]. Although the potentially important role of HO-1 in human milk, only one previous study has investigated the concentration of HO-1 in the HM of healthy mothers having delivered at term of Gestational Age.

The present study will evaluate (a) the pattern of HO-1 in preterm and term human milk, over the transition from colostrum to mature milk; (b) the concentration of HO-1 in donor milk, to evaluate the possible effect of HoP on this protein.

Methods

We performed a pre-test/test study, where milk donors acted as their own controls. Mothers admitted into the study gave signed and informed consent. The study protocol was approved by the local Ethic Committee of the Italian Association of Human Milk Donor Banks (AIBLUD).

We enrolled 14 healthy mothers: 7 who have delivered at term (Gestational age >37 weeks) and 7 who have delivered preterm (Gestational age <37 weeks) [20]. Samples were collected from colostrum 48h after birth, from transition milk on Days 7 and 14, and from mature milk on Day 30 after birth (colostrum: n=14; transition: n=14; mature: n=14, respectively) [21]. Standard exclusion criteria for human milk donation set forth by AIBLUD guidelines were applied [4]. Moreover we excluded also: multiple pregnancies, any CNS illness, gestational diabetes and hypertension, systemic infection, intrauterine growth retardation, cardiac or metabolic or hemolytic disease, malnutrition and maternal allergy and fetuses with any malformation and/or chromosomal abnormalities.

Collection and Pasteurization of Human Milk

Fresh milk samples were collected at the same time (9-10 AM) into sterile, disposable, high-density polyethylene sealed bottles (Flormed, Naples, Italy). Milk was collected with standard extraction methods by means of an electric breast pump (Medela Symphony, Baar, Switzerland). According to current guidelines and in order to collect full pumping samples, the extraction session was stopped 2 min after the outflow of the last drops of milk [4,22]. From the total amount of milk of each mother, a sample of 10 mL of milk was collected and then further divided into two parts. The first was immediately frozen at -80°C (NO-HoP), while the second part was pasteurized and then frozen at -80°C (HoP).

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.

Heme Oxygenase-1 Measurements

Samples were immediately stored at -80°C until analysis. Heme oxygenase-1 levels were determined using a specific ELISA test (SL0838HU-97 Human heme oxygenase 1 ELISA) according to the manufacturer's instructions (SungLong Biotech Co., Ltd.; China). Investigators who performed the laboratory tests were blind to storage modalities. The assay detection limit is 1.00 ng/ml, the coefficient of variability intra-assay is $\leq$ 5.0% and the coefficient of variability inter-assay is $\leq$ 10%. The assay is specific for Heme oxygenase-1.

Statistical Analysis

Demographic characteristics of maternal and neonatal outcomes were reported as mean ± SD. HO-1 concentrations were expressed as median and interquartile ranges. Statistical analysis was performed by using two-tiled paired t-test and by Mann–Whitney two-sided U-test when data did not follow a Gaussian distribution. Comparison between groups was performed by using ANOVA one-way test. A $P<0.05$ was considered significant.

Results

Maternal and perinatal characteristics of milk donors are reported in Table 1.

HO-1 was detectable in all human milk samples. Its concentration was higher in colostrum (median 74.8 ng/ml; 25° centile 59.1 ng/ml and 75° centile 83.7 ng/ml) with respect to transition milk (median 64.2 ng/ml; 25° centile 52.9 ng/ml and 75° centile 77.7 ng/ml) and to mature milk (median 72.2 ng/ml; 25° centile 71.4 ng/ml and 75° centile 86.6 ng/ml): nonetheless, the differences in HO-1 concentration were not significant, as represented in Figure 1 ($P>0.05$).

After correction for gestational age, we can conclude that HO-1 does not significantly differ between preterm (median 69.4 ng/ml; 25° centile 53.7 ng/ml and 75° centile 71.6 ng/ml) and term milk samples (median 75.5 ng/ml; 25° centile 58.8 ng/ml and 75° centile 83.7 ng/ml), $P>0.05$ (Figure 2).

HO-1 was detectable also in all pasteurized milk samples,

Parameter	n=14
Mean (± SD) maternal age, years	35 ± 5
Parity 1 n. (total)	10 (14)
Mode of delivery, n. Caesarean Vaginal	 5 9
Mean (± SD) gestational age, weeks	36 ± 4
Mean (± SD) birth weight, g	2950 ± 1100
Gender male (female)	6 (8)

Table 1. *Demographic characteristics of milk donors*

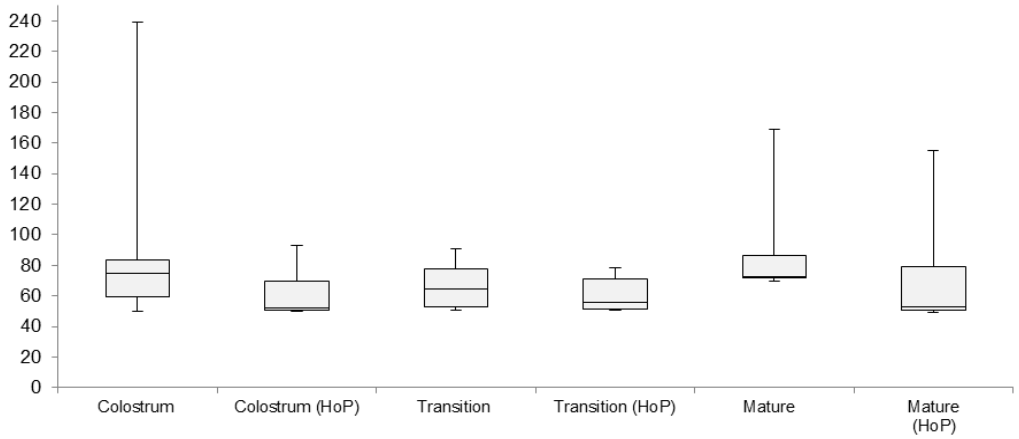

Figure 1. *Heme oxygenase-1 concentrations (ng/mL) in milk samples during maturation degree, before and after HoP*
The lower and upper bars represent the 5° centile and 95° centile, respectively; interquartile range is indicated by the box and median value is represented by the horizontal line in the box. No significant differences have been found between studied groups (P>0.05).

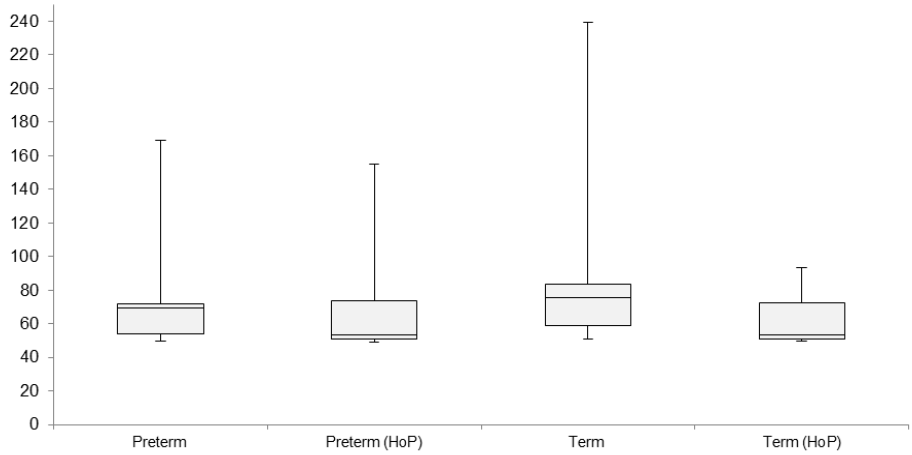

Figure 2. *Heme oxygenase-1 concentrations (ng/mL) in preterm and term samples, before and after HoP*
The lower and upper bars represent the 5° centile and 95° centile, respectively; interquartile range is indicated by the box and median value is represented by the horizontal line in the box. No significant differences have been found between studied groups (P>0.05).

as shown in Figure 1. No significant differences in HO-1 levels were observed in all milk samples before HoP (median 71.4 ng/ml; 25° centile 57.0 ng/ml and 75° centile 83.5 ng/ml) and after HoP (median 53.7 ng/ml; 25° centile 50.7 ng/ml and 75° centile 73.8 ng/ml) (P>0.05). No significant differences (P>0.05) have been found between groups when HO-1 concentrations were compared sub-grouping for gestational age (term vs. preterm) (Figure 2). Furthermore, HO-1 levels did not differ (P>0.05, for both) in both groups when compared for the degree of milk maturation (Figure 1).

Discussion

Human milk contains several biological factors that are involved in a newborn's growth and immune system regulation, and which are fundamental to ensure protection against infection and to prevent NEC in preterm newborns [1,23]. Among these factors, HO-1 is an antioxidant protein and an enzyme of the Heat Shock Protein group which has been associated to important cytoprotective actions [23-27].

Our study is the first to show that HO-1 is present in preterm human milk and in donor milk. No statistically significant differences were found between preterm and term milk samples; likewise, our results showed a not significant decrease in HO-1 levels as the milk matures, in both term and preterm milk. These findings are in agreement with the notion that the concentrations of colostrum and milk constituents change with suckling time. Only one study was conducted previously in order to evaluate the presence of HO-1 in term human milk [28]. Our data are in agreement with the results of Li Volti although we did not find significant differences between colostrum and mature milk [28]. The presence of a typical intracellular protein such as HO-1 in milk is consistent with previous observations demonstrating the presence of other intracellular proteins (e.g. α-lactalbumin, calmodulin and osteocalcin) in extracellular space and/or biological fluids. The presence of such proteins in milk is related to an active secretory mechanism and/or to the anatomical and physiological characteristics of the mammary gland (i.e., apocrine mechanism of secretion).

Moreover our study is the first that evaluates the effect of HoP on HO-1 levels in human milk. The data show that this heat treatment does not affect significantly HO-1 concentrations also after correction for gestational age and maturation degree of the human milk. Currently, Holder pasteurization (62.5°C for 30 min) is the most studied

and recommended method for the heat treatment of donor human milk in Human Milk Banks, since it represents a good compromise between microbiological safety and nutritional and biological quality of the human milk [4,5]. In fact, this method is able to inactivate milk pathogens but partially degrades immunologic and anti-infective factors such as IgA, IgG, lysozyme, lactoferrin, lymphocytes, growth factors and lipase as well [29,30]. Nonetheless, with Holder pasteurization other key nutritional factors (i.e., oligosaccharides, lactose, LCPUFA, fatty acids and vitamins) remain unaffected [29,31]. The loss of biological factors through pasteurization may have significant implications and this is particularly important in preterm infants with an immature immune system, who are at increased risk of developing Necrotizing Enterocolitis (NEC).

In particular milk HO-1, similarly to other milk antioxidant enzymes, may exert protection via its antioxidant activity, which is related to the conversion of free heme into three end products: (i) biliverdin, which is rapidly reduced into bilirubin (which itself possesses antioxidant activity) [32]; (ii) carbon monoxide, a potential gaseous neurotransmitter and vasodilator with anti-inflammatory and antiapoptotic activities [17]; and (iii) iron (Fe^{2+}), which is sequestered by iron-binding proteins [33,34]. Moreover HO-1 has probably an immunoregulatory role that is not only dependent on its enzymatic activity but is related to its ability to bind specific receptors, thus regulating the immune response. In fact extracellular stress proteins, including HSP, are emerging as important mediators of intercellular signaling and transport [35,36]. Release of such proteins from cells is triggered by behavioral stress, as well as by exposure to immunological "danger signals" [34,37]. After release into extracellular fluid, HSP may then bind to the surfaces of adjacent cells and initiate signal transduction cascades, as well as transport of cargo molecules such as antigenic peptides and chaperokines with an immunomodulatory effect [16]. In particular, Li Volti et al. using a molecular modeling approach, found that an important immunoregulatory receptor (i.e., CD91) is the possible ligands of HO-1. Nevertheless, integrating experimental data with in silico data also highlighted the putative role for HO-1 as an immunomodulatory molecule [28].

Bearing in mind the different functions of HO-1 in several tissue, the data reported in literature by four meta-analysis showing a reduction in NEC incidence in preterm babies fed with donor milk compared to those fed with preterm formula and the unclear pathophysiology of NEC (immature gastrointestinal epithelium, impaired immunological defences, enteral feeding and bacterial colonization), it is possible to argue that human milk HO-1 may play a role in development and regulation of immune system of the gastroenteric tract [6-9].

Conclusion

Our study showed that HO-1 is present not only in term human milk but also in preterm human milk in similar concentrations. Additionally, our data suggest that the biological value of human milk associated with the HO-1 content is maintained after Holder pasteurization. Further data concerning the metabolic fate of HO-1 in the gastro-enteric tract is needed to corroborate the hypothesis that HO-1 participates in the nutritional and immunoregulatory effects of milk.

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