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Effect of pasteurization on melatonin concentration in human breast milk

Original Study

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Abstract

Introduction. Women who have problems with lactation can use human milk banks. Mainly this human milk is provided to premature babies and sick newborns. Human milk is the most suitable food for newborns and infants, recommended by WHO (World Health Organization). Human milk has anti-inflammatory, anti-infective, and anti-allergic properties, and also works for immunomodulation. Melatonin has a special, underestimated importance in the composition of breast milk. It is a hormone that has many body functions and, for several decades, its antioxidant potential has been increasingly talked about.
The aim of the study was to examine the effect of Holder pasteurization on melatonin concentration in human milk.
Materials and Methods. 18 samples of human milk from donors from the human milk bank were used for the analysis. Melatonin concentration before and after pasteurization was determined by ELISA. In addition, the nutritional content composition of milk was analyzed using MIRIS Human Milk Analyzer and correlations examined.
Results. Melatonin concentration in human milk before pasteurization was 0.65-26.24 pg/mL (Me=9.58, IQR=12.72), while after pasteurization 0.80-29.58 pg/mL (Me=9.98, IQR=11.26). There was a positive correlation between melatonin concentration before and after pasteurization (r=0.797, p<0.001).
Conclusions. The Holder pasteurization process does not affect the concentration of melatonin in milk samples, which may be a recommendation for human milk banks.

Keywords

melatonin • Holder pasteurization • human milk • donor milk • human milk bank • lactation • newborn

1. Introduction

Breastfeeding is recommended by WHO (World Health Organization) and UNICEF (United Nations International Children's Emergency Fund) due to the health benefits for both mother and child. WHO recommends exclusive breast milk feeding up to 6 months of age, later continuing breastfeeding and complementary nutrition, preferably up to 2 years or more if mother and child have such a need [1]. Breastfeeding reduces the risk of children's gastrointestinal infections, respiratory tract infections, otitis media, bacterial otitis media, sepsis, urinary tract infections, sudden infant death syndrome, type I and type II diabetes, overweight, obesity, lymphoma, leukemia, hypercholesterolemia, allergic diseases, and also allows for a milder course of the above diseases [2].

The lactation phase and other factors such as the woman's health, physiology, race, and environment, determined the composition of milk; diet has a small effect on its composition [3]. Milk consists of proteins, carbohydrates, fats, vitamins, hormones, immunoglobulins, growth factors, cytokines, chemokines, mother's blood cells, and other biologically active compounds [4-7].

Melatonin (N-acetyl-5-methoxytryptamine), an endogenously produced indoleamine, is an underrated and nonspecific component of breast milk. This hormone is synthesized mainly by the pineal gland, as well as through the digestive tract, ovaries, retina, thymus, bone marrow, and brain. It regulates the rhythm of sleep and wakefulness, and has anti-inflammatory and antibacterial effects, protecting the child from parasites. It is also a powerful antioxidant [8, 9]. Scientific research suggests that it may play an important role, due to its antioxidant activity, in many diseases, such as neurodegenerative diseases (Parkinson's disease, Alzheimer's disease, multiple sclerosis) [10], diabetes [11], gastrointestinal diseases (peptic ulcer, reflux disease,

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irritable bowel syndrome) [12], and cardiovascular disease (hypertension) [13]. It is also said to have anti-cancer properties [14]. In human milk, it plays an important role, in addition to regulating the child's sleep and wakefulness; it probably affects the development of the digestive system and alleviates the occurrence of colic [15]. It is important to recognize that the fetus does not secrete melatonin until 9–15 weeks of age. Levels increase up to the third trimester of gestation and drop after parturition. However, a mature circadian rhythm and response to the light/dark cycle develops at 2–3 months of life. Thus, human milk is the only melatonin source in the perinatal period, and the infant melatonin level depends in this period on the circadian melatonin rhythm of the mother [16]. Some studies have attributed longer sleep time in breastfed infants compared with that in formula-fed infants to melatonin in breast milk, as no melatonin is detected in formula [17].

For sick and premature babies, or if mother's milk (MOM) is not available or is insufficient, human donor milk from a milk bank is recommended.

There are 248 human milk banks in Europe, including 16 in Poland [18]. The role of the institution is to recruit donors (mothers who breastfeed their own child and still have surplus of milk); test donors; and to collect, preserve, and distribute human milk to premature babies in the NICU. In order for banked MOM to be suitable for transmission to a child, it must undergo bacteriological screening and pasteurization. Donor milk in milk banks undergoes various procedures, including collection, freezing, thawing, and pasteurization to ensure microbiological purity. Despite the search for alternatives to the thermal methods of preserving breast milk, pasteurization by the Holder method is the most commonly used [19, 20]. Pasteurization is a process of heating breast milk to the right temperature. The most commonly used is Holder pasteurization (HoP), otherwise known as LTLT (62.5 °C, 30 min, low temperature, long time), to inactivate pathogens such as bacteria, viruses, or parasites. According to scientific data, the process effectively inactivates, among others, the Zika virus and HPV [1]. However, heat treatment can reduce the content of bioactive compounds in breast milk [21, 22]. Therefore, the purpose of this study was to investigate the effect of pasteurization on melatonin concentration in human milk, and thus to draw attention to the importance of the presence of human milk banks in the world.

2. Materials and Methods

Upon entering the study, donors signed an informed consent and were provided with verbal and written instruction for milk sample collection.

18 samples of human milk were collected from 10 mothers donating to the Human Milk Bank at the Ludwik Rydygier Provincial Polyclinical Hospital in Toruń, Poland. The inclusion criteria were: recruited in Human Milk Bank at the Ludwik Rydygier Provincial Polyclinical Hospital in Toruń, qualified as a milk donor in accordance with the requirements and protocol of the human milk bank, exclusive breastfeeding, and with surplus of milk.

Samples were divided into pre- and post-pasteurization aliquots and were Holder pasteurized.

Breast milk for the analysis was expressed into sterile containers dedicated to storage of human milk. The unprocessed milk was tested for macronutrient content. Samples were stored at -80 °C until melatonin determination. Clinical data (age, lactation period, and gestational age (g.a.) were collected from the nursing mothers.

2.1. Determination of the basic composition of breast milk

MIRIS Human Milk Analyzer (Miris AB, Uppsala, Sweden) was used to analyze macronutrients (total fat [g/100 mL], carbohydrates [g/100 mL], protein [g/100 mL], total solids [g/100 mL], and energy content [kcal/100 mL]) in milk samples. The Miris HMA is based on semi-solid mid-infrared (MIR) transmission spectroscopy. The wave ranges used in the device are specific for different groups: carbonyl (5.7 µm) for fat, amide groups (6.5 µm) for protein, and hydroxyl groups (9.6 µm) for carbohydrate. A daily calibration check was performed prior to analysis using the calibration solution provided by the supplier.

Each sample before analysis was heated at 40 °C in a thermostatic bath and then homogenized using the Miris Sonicator [1, 5 sec/mL]. Each sample was analyzed in triplicate.

2.2. Holder pasteurization

Holder pasteurization of human milk samples was done at the Human Milk Bank at the Ludwik Rydygier Provincial Polyclinical Hospital in Toruń, Poland on automatic Human Milk Pasteurizer S90 (Sterifeed, MEDICARE COLGATE LTG). Samples of 50 mL were treated according to Regional Human Milk Bank standard pasteurization protocol at 62.5 °C for 30 min. followed directly by rapid refrigerated cooling to less than 4 °C. The correctness of the process was confirmed with the data logging system, by recording the temperature of the bottle probe every minute.

2.3. Determination of melatonin concentration in breast milk

To determine the concentration of melatonin in milk before and after pasteurization, an enzyme-linked immunoassay (Melatonin ELISA, IBL International) was used. 0.5 mL samples, melatonin standards and reagents available from the manufacturer, melatonin extraction columns and a coated 96-well plate were used for the analysis. Extraction of samples was carried out according to the manufacturer's data and extracts were prepared. 100 µl ready extracts were applied to a 96-well plate and incubated according to the manufacturer's instructions. The result was read using a reader (MULTISKAN GO, Thermoscientific) at 405 nm wavelength. Limit of detection was 0.6 pg/mL.

We had the consent of the Bioethics Committee (No. KB 437/2018) of the Nicolaus Copernicus University in Toruń at the Collegium Medicum in Ludwik Rydygier in Bydgoszcz.

2.4. Statistical analysis

For statistical analysis, the Statistica 13.1 software package from StatSoft® was used (StatSoft Poland, Kraków). The normality of the distribution was verified by the Shapiro-Wilk test. There was no normality in the distribution of quantitative variables analyzed. Parameter variability is presented in the form of median, minimum and maximum values (min-max), and interquartile range (IQR). In order to assess the strength of the relationship between the studied parameters, the Spearman rank correlation test was used. The results at the level of $p<0.05$ were considered statistically significant. The non-parametric Wilcoxon paired test was used to assess statistical significance in two groups of dependent variables without normality of distribution.

3. Results

3.1. Characteristics of analyzed group of milk samples

The study population was characterized by a mean age of 29.34±3.97 years, gestational age at birth of 35.06±5.06 weeks, mean lactation period 14.44±4.96 week (Table 1).

3.2. Melatonin concentration and the macronutrient composition of milk

Melatonin concentration in milk samples before pasteurization was 0.65-26.24 pg/mL (Me=9.58, IQR=12.72), while after pasteurization it was 0.80-29.58 pg/mL (Me=9.98, IQR=11.26).

The macronutrient composition of milk is presented in Table 2. Positive correlations were observed between: lactation period and g.a. ($r=0.687$, $p=0.001$), fat content and dry matter ($r=0.789$, $p<0.001$), fat content and energy value ($r=0.945$, $p<0.001$), total protein and dry matter ($r=0.482$, $p=0.031$), total protein and energy value ($r=0.459$, $p=0.042$) and energy value and dry matter ($r=0.871$, $p<0.001$), while the negative correlation between the lactation period and total protein content ($r=-0.589$, $p<0,001$) (Table 2).

3.3. The effect of pasteurization on melatonin concentration in human milk

There were no statistically significant differences between melatonin concentration before pasteurization and melatonin concentration after pasteurization ($p=0.085$) (Fig. 1). There was a positive correlation between melatonin concentration before and after pasteurization ($r=0.797$, $p<0.001$) (Fig. 2, Table 2).

4. Discussion

The functioning of milk banks in the world is particularly important for premature and sick infants, due to the content of biologically active components in milk that support the immune system and digestive system [19, 21, 22, 23]. Human milk banks obtain milk from volunteers. Then, in order to ensure microbiological purity, the Holder pasteurization process takes place, and the samples prepared in this way are frozen and await collection. The process of pasteurizing human milk at human milk banks raises questions due to the possibility of a reduction of bioactive ingredients. Therefore, for several decades, much attention has been paid to it.

A very important, underrated hormone in human milk is melatonin, which, in addition to regulating sleep and wakefulness in children, is a powerful antioxidant. Additionally, there are

Table 1. Characteristics of tested milk samples with basic composition

Variable	Median	Min	Max	Interquartile range (IQR)
Age [year]	29.00	24.00	37.00	7.00
Lactation period [week]	12.00	3.00	40.00	14.00
Gestational age [week]	36.5	27.00	41.00	8.00
Melatonin pre-pasteurization [pg/mL]	9.58	0.65	26.24	12.72
Melatonin post-pasteurization [pg/mL]	9.98	0.80	29.58	11.26
Fat [g/100 mL]	2.95	1.70	3.80	0.60
Total protein [g/100 mL]	1.40	1.20	1.90	0.25
Carbohydrate [g/100 mL]	7.70	6.70	7.90	0.25
Dry matter [g/100 mL]	12.30	11.00	13.10	0.90
Energy value [kcal/100 mL]	65.50	54.00	72.00	6.00

Table 2. Correlations of the tested parameters according to Spearman's rank order

	Age [year]	Gestational age [week]	Lactation period [week]	Melatonin pre-pasteuri-zation [pg/mL]	Melatonin post-pasteur-ization [pg/mL]	Fat [g/100 mL]	Total protein [g/100 mL]	Carbohydrate [g/100 mL]	Dry matter [g/100 mL]	Energy value [kcal/100 mL]
Age [year]	1	0.329	0.276	0.068	-0.102	0.113	-0.052	-0.044	0.144	0.092
Lactation period [week]	0.276	0.687*	1	-0.263	-0.255	-0.175	-0.589*	0.013	-0.062	0.071
Melatonin pre-pasteurization [pg/mL]	0.068	-0.290	-0.263	1	0.797*	-0.043	0.099	0.237	0.075	-0.104
Melatonin post-pasteurization [pg/mL]	-0.102	-0.248	-0.255	0.797*	1	-0.128	0.300	-0.019	0.028	-0.127
Fat [g/100 mL]	0.113	0.173	-0.175	-0.043	-0.128	1	0.215	-0.153	0.789*	0.946*
Total protein [g/100 mL]	-0.052	-0.215	-0.589*	0.099	0.300	0.215	1	-0.121	0.482*	0.459*
Carbohydrate [g/100 mL]	-0.044	0.156	0.013	0.237	-0.019	-0.153	-0.121	1	0.211	-0.144
Dry matter [g/100 mL]	0.144	0.157	-0.062	0.075	0.028	0.789*	0.482*	0.211	1	0.871*
Energy value [kcal/100 mL]	0.092	0.178	0.071	-0.104	-0.127	0.946*	0.459*	-0.144	0.871*	1

**correlation coefficients are significant with p<0.050*

reports of its beneficial effects on the child's digestive system and on soothing the collections [15].

The effect of pasteurization on hormones has been insufficiently studied, so it is worth extending the research to this issue because of their significant role. There are few studies concerning melatonin in breast milk soon after birth and the evolution of melatonin levels during the lactation period under normal conditions [16].

To our knowledge, this is the first scientific study to compare the concentration of melatonin in human milk before and after Holder pasteurization. However, the concentration of melatonin in cow's milk and ultra-high temperature (UHT) processed milk were compared. Although the milk was subjected to 140 °C for 2 s, the melatonin content was not significantly different in fresh milk (4.08 pg/mL) and UHT milk (4.16 pg/mL), implying that the UHT process does not significantly change the melatonin content in cow's milk [24].

Our studies have shown that the concentration of melatonin after pasteurization (0.80–29.58 pg/mL [Me=9.98, IQR=11.26]) does not significantly differ from the concentration before pasteurization (0.65–26.24 pg/mL [Me=9.58, IQR=12.72]). In addition, a positive correlation between melatonin concentration before and after pasteurization was observed ($r=0.797$, $p<0.001$). Such results are valuable information regarding the use of the pasteurization process for milk banks. At present, there are few scientific reports on the concentration of melatonin in human milk and its function for the proper development of the child [16, 17, 19].

Another hormone with circadian variation in human milk is cortisol, which has a beneficial effect on the digestive tract of premature babies [25, 26, 27]. Researchers suggest that cortisol levels in breast milk have an impact on infant behavior. It was observed that children (male sex) 3 months after delivery with a higher concentration of this hormone were more confident, bold, active, and interested in the world [28], while children (female sex) with lower levels of this hormone were more prone to fear, sadness, discomfort, and frustration [29]. According to Voorn et al. (2017) and other authors, pasteurization does not significantly affect cortisol and cortisone levels in breast milk [27, 30].

However, scientific data suggest that the pasteurization process carried out in human milk banks may affect the concentration and activity of the food components [5, 16, 31, 32]. Guerra et al. (2018) examined the effect of Holder pasteurization (62.5 °C, 30 min.) on the protein profile and lysozyme (LZ) activity in human milk. They showed that Holder pasteurization does not affect LZ activity, but that it reduces glutathione peroxidase (GPx), which is an important antioxidant that can affect the modulation of a child's intestinal microflora. In addition, a reduction in the content of immunoglobulins and lactoferrins has been observed [33]. Silvestre et al. (2008) showed that pasteurization reduces

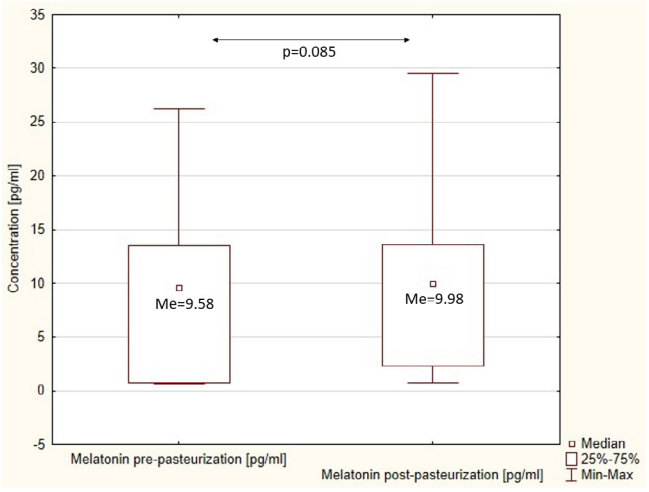


Fig. 1. Comparing melatonin concentration before and after Holder pasteurization

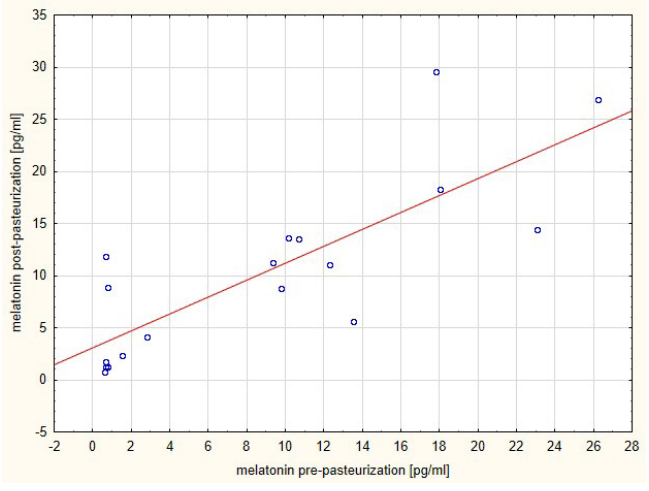


Fig. 2. Correlation between melatonin concentration before pasteurization and melatonin concentration after pasteurization (Spearman's rank correlation)

the overall antioxidant capacity of milk, while the opposite result was obtained by Elisia and Kitts (2011) [34, 35].

Pasteurization of human milk affects to varying degrees the kinetics of proteolysis during gastrointestinal digestion [4]. This process influences the peptidome of human milk before digestion, mainly derived from β-casein, which results in the induction of other kinetics of peptide release during gastrointestinal disintegration [36]. It has been shown that heat treatment below 62.5 °C has a minimal effect on lactoferrin, sIgA, lysozyme, while above that temperature the proteins are inactivated faster [32].

Peila et al. (2016) examined the effect of pasteurization on the protein profile and showed that in most samples this process did not cause modification in the profile, and thus did not affect the activity of human milk proteins [37].

Subsequent researchers have observed that pasteurization can affect the content of vitamins and microelements [31, 38,

39]. It reduces the content of vitamin C and B6, while it has no effect on vitamin D, B2, B12, and E [39].-After a given process, there was a significant decrease in Ca (259.4 ± 96.8 to 217.0 ± 54.9), P (139.1 ± 51.7 to 116.8 ± 33.3) and K (580.8 ± 177.1 to 470.9 ± 109.4) [31].

A group of scientists has shown that pasteurization does not significantly affect some components of human milk [7, 40, 41, 42]. Such components are, among others, activin A [41], glycosaminoglycans (GAG) [40, 42], some cytokines (IL-2, IL-4, IL-5, IL-13, IL-17), growth factors (EGF, TGFβ1, TGFβ2), lipids, polyunsaturated fatty acid, monounsaturated fatty acid [30], oligosaccharides [7], myoinositol, lactose, and oxidative stress markers such as malondialdehyde and Oxygen Radical Absorbance Capacity (ORAC) [42]. Mohd-Taufek et al., (2016) did not observe significant differences between the studied elements (zinc, copper, selenium, magnesium, iron, iodine, molybdenum, bromine) after the pasteurization process except for iron [43].

Due to numerous studies that confirm the adverse effects of Holder pasteurization on human milk constituents, milk banks are looking for alternative preservation methods. HTST and HPP technologies appear to be the most promising. International studies suggest that high temperature short time (HTST) was effective against *Escherichia coli, Staphylococcus aureus, Streptococcus* A and B, and HIV, while at the same time preserving the nutritional composition of human milk [44]. None of the pasteurization technologies had a significant effect on TGF-β2, EGF, adiponectin and ghrelin concentrations. [4]. Klotz et al (2017), showed that HTST samples compared to HoP–processed samples retained higher rates of the following parameters: immunoglobulin A (95% vs. 83%); alkaline phosphatase (6% vs. 0%), and bile salt-stimulated lipase (0.8% vs. 0.4%) [45].

Another method is the high-pressure, non-thermal technology HPP. This process effectively eliminates Gram-negative and Gram-positive bacteria depending on the pressure applied. The process effectively eliminates, for example, *Listeria monocytogenes*, *Eschericha coli*, *Staphylococcus aureus*, *Staphylococcus agalactiae*, *Salmonella spp* and *Bacillus cereus*. Additionally viruses such as HIV and CMV are inactivated [46]. In the study of Wesołowska et al. (2108) with the application of 4 pressure variants for the optimal variant in comparison with the Holder method, a number of advantages were demonstrated during pasteurization: an increase in leptin 90.01% vs. 77.86%; adiponectin 100% vs. 32.79%, IgG 82.24% vs. 49.0.4%. Lactoferrin, insulin, and HGF levels were higher in HPP compared to HoP. It is suggested that the HPP 200 MPa + 400 MPa variant may be the best method of maintaining the most suitable human milk composition [22]. HPP does not deactivate free fatty acids and has no significant effect on the vitamin C and A content of human milk [38]. Demazeau et al. (2018) showed that IgA activity at 400 MPa does not change, while in the other range concentration of immunoglobulin changes slightly [28].

Our study has several strengths and limitations. Above all, this is the first study to research the concentration of melatonin before and after Holder pasteurization (HoP) and second, has shown that HoP does not reduce melatonin concentration in breast milk.

A limitation of the study was the missing information about the study group, such as the weight of the women, the method of getting pregnant, the weight of the newborns, the presence of diseases, possible childbirth complications, the number of boys and girls. This should be considered in future studies. Storage conditions before HoP should be included, too. As shown by Molad et al. (2019), after 24 hours in a refrigerator, water evaporation from the milk occurs, which implies higher melatonin levels [19]. Also, the study was designed as a feasibility study, so the sample size was relatively small. We suppose that a larger milk sample size and pooled human milk from donors should have been used. This would give more power and relevance to the findings of the study, which may be recommended for milk banks.

5. Conclusions

Melatonin concentration in breast milk before pasteurization was 0.65–26.24 pg/mL (Me=9.58, IQR=12.72), while after pasteurization it was 0.80–29.58 pg/mL (Me=9.98, IQR=11.26). These results may have value regarding the use of HoV for human milk banks. Further research is needed to evaluate the influence of other factors not taken into account in this study.

Authors' Contribution

A.C.: data analysis and interpretation, writing – original draft preparation, literature review, final proofreading and approval of the version for publication; **E.S.D.**: research concept and design, acquisition of data, data analysis and interpretation; **M.L.**: supervising the project, carrying out the experiments; **D.O.S.**: writing – review and editing; **B.S.**: research concept and design, literature review; **K.L.**: data analysis

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Conflict of Interest

The authors have no potential conflicts of interest to declare.

Ethics Approval

We had the consent of the Bioethics Committee (No. KB 437/2018) of the Nicolaus Copernicus University in Toruń at the Collegium Medicum in Ludwik Rydygier in Bydgoszcz.

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